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RIVISTA MENSILE DI SCIENZE PURE E APPLICATE
MONTHLY JOURNAL OF PURE AND APPLIED SCIENCE

Editores:

P. HUBER · R. MATTHEY · A. v. MURALT · L. RUZICKA
Basel Lausanne Bern Zürich

Redactor: H. MISLIN, Basel-Mainz

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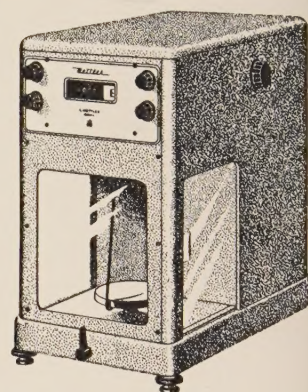
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L'EXPERIENTIA paraît le 15 de chaque mois. Vente et abonnement dans toutes les librairies suisses et étrangères, ou directement chez l'éditeur. Abonnement pour un an Fr. 44.- pour la Suisse; pour l'étranger Fr. 56.-. Ces prix s'entendent en francs suisses.

Die EXPERIENTIA erscheint am 15. jedes Monats und kann im In- und Ausland durch jede Buchhandlung oder direkt vom Verlag bezogen werden. Der Abonnementspreis beträgt in der Schweiz Fr. 44.-, im Ausland sFr. 56.-.

Insertionspreise: $\frac{1}{1}$ Seite Fr. 220.-, $\frac{1}{2}$ Seite Fr. 132.-, $\frac{1}{4}$ Seite Fr. 77.-. Inseratenannahme durch den Verlag.

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EXPERIENTIA is published on the 15th of every month and can be obtained in any country through the booksellers or from the publishers. The price by annual subscription by inland mail is fr. 44.-; other countries sfr. 56.-. Prices in Swiss currency.

Prices for advertising: $\frac{1}{1}$ page fr. 220.-, $\frac{1}{2}$ page fr. 132.-, $\frac{1}{4}$ page fr. 77.-. Advertisements should be sent to the publishers.

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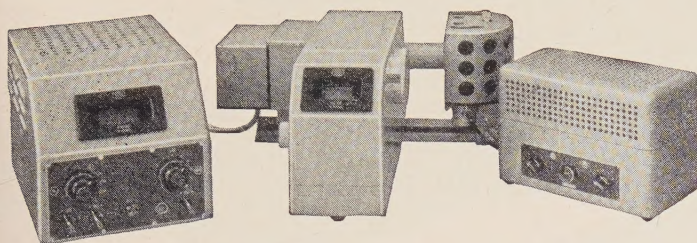
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Tumor Isoimmunity¹

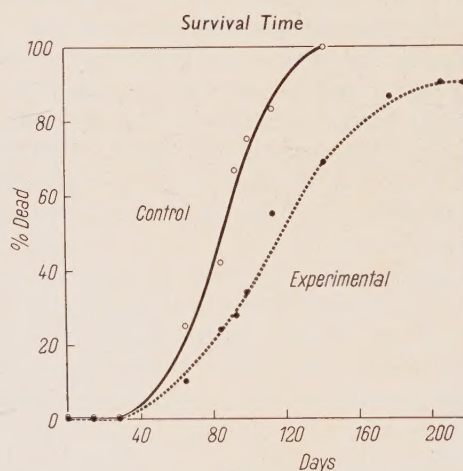
By H. M. HIRSCH²

The problem of immunity in inbred animals against their own tumors has been a central one in experimental cancer research for many years. Its importance is due to the fact that the problem of tumor isoimmunity and tumor autoimmunity centers around the question of specific antigenic components present in tumor and absent in homologous normal tissue. A rational approach to tumor therapy by immunological means clearly hinges on this central point. Despite the many papers written on the subject, the desiderata for a critical attack on the problem have not always been realized³, mainly because a clear distinction between isoimmunity, autoimmunity, and homograft immunity⁴ has not frequently been made. Thus, as will be pointed out below, the relevance of much of the work involving long-transplanted or other unsuitable tumors, or unsuitable hosts, to the problem of isoimmunity, and of induced immunity against spontaneous tumor development, remains problematical.

No reference will be made here to the dilemmas which plagued the early workers in the field of tumor immunity and which we now know were clearly ascribable to phenomena involving homotransplantation reactions in genetically heterogeneous populations; this aspect of tumor immunity has been well summarized elsewhere (see, e.g., MEDAWAR's Harvey Lecture⁵). We shall be dealing here, rather, with work

which clearly lies or has been assumed to lie within the province of tumor isoimmunity.

Recent experiments by HIRSCH *et al.*³ have demonstrated a slight but definite amount of immunity in strain C (Bagg albino) mice against a homologous challenging tumor of recent spontaneous origin, following a course of immunizations with actively growing tumor of indigenous origin. No differences in number of tumors or in the time at which the tumors appeared were apparent, and immunity expressed itself only in a significantly longer survival time of the treated animals as compared with the control group. Some of the data obtained are summarized in the Figure.



Survival time of immunized and non-immunized strain C (Bagg albino) mice following challenge with strain C tumor. Tumor used for immunization: Strain C tumor in first transplant generation. Mean survival time for experimental group, 124 ± 8.7 days, for control group, 91 ± 7.3 days; $P = < 0.01$. For experimental details, see ⁶.

These results tend to indicate that an antigenic difference exists between host and tumor tissue. A number of definite reservations have been pointed out⁶ which must be considered before this view can be fully accepted. Such a view is, however, in accordance with preliminary data published by ISOJIMA *et al.*⁷ and with findings obtained by methods employing anaphylactic phenomena by GRACE⁸, and by ZIL'

¹ Assisted by grants from the National Cancer Institute of the National Institutes of Health, Public Health Service; the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council; and the Minnesota Division of the American Cancer Society.

² Scholar in Cancer Research of the American Cancer Society. Division of Cancer Biology, Department of Pathology, University of Minnesota Medical School, Minneapolis.

³ H. M. HIRSCH, J. J. BITTNER, H. COLE, and I. IVERSEN, *Bact. Proc.* 1958, 60; *Cancer Res.* 18, 344 (1958).

⁴ For purposes of discussion, the terms autoimmunity, isoimmunity, and homograft immunity will be defined in this manner: autoimmunity—immunity elicited in an animal against his own normal or tumor tissue; isoimmunity—immunity elicited in a member of an inbred strain against normal or tumor tissue of another member of the same inbred strain; homograft immunity—immunity elicited in an individual against normal or tumor tissue of a genetically different individual of the same species, or that elicited in animals of one inbred strain against normal or tumor tissue of members of other inbred strains of the same species.

⁵ P. B. MEDAWAR, *The Harvey Lectures*, 1956-1957 (Academic Press, Inc., New York 1958), p. 144.

⁶ H. M. HIRSCH, J. J. BITTNER, H. COLE, and I. IVERSEN, *Cancer Res.* 18, 344 (1958).

⁷ S. ISOJIMA, R. M. GRAHAM, and J. B. GRAHAM, *Proc. Amer. Ass. Cancer Res.* 2, 310 (1958).

⁸ J. T. GRACE, JR., Personal communication.

BER⁹ (see this paper for a review of pertinent earlier work).

A brief attempt will be made to correlate and interpret the facts described above as well as those found in the literature (for review see ¹⁰), always keeping in mind the strictures alluded to above.

The host does develop immunity against tumors indigenous to his own strain, and possibly even against his own tumor¹¹, but this immunity is slight and may be overcome by the independent and aggressive behaviour of the tumor. It would seem that HAALAND's dictum 'an animal cannot be immunized against its own neoplastic cells' may, like most dicta, have been too categorical.

Host-tumor interplay must thus be viewed as a phenomenon whose outcome hangs in the balance, but one which is prejudiced in favor of the tumor. Immunization will work the better, the further removed the tumor is from the original host¹², a condition which is facilitated by continued transfer of the tumor, and repeated immunization bolsters the host's immune response¹³. Conversely, the closer the tumor is to the host, genetically, the less the immunity⁶. In fact, as far as transplantability in the original host is concerned, work involving long-transplanted tumors can essentially be considered a problem in homotransplantation rather than isotransplantation. Prolonged serial transplantation of the tumor is not always a necessary prerequisite for such changes in the tumor to occur, and changes in transplantability characteristics may be accompanied by changes in the ploidy of the tumor¹⁴. On the other hand, the host's immune response, even if once well established, can be overcome by too great a challenge dose¹⁵.

Additional evidence for the view that use of long-transplanted tumors elicits homograft, rather than isograft, reactions with respect to the original host comes from work comparing immune responses of inbred animals against indigenous tumors and against tumors induced by carcinogenic agents. FOLEY¹⁶ has shown that, following removal of transplantable tumors of recent spontaneous origin (mammary adenocarcinomas), no immunity was found to be present under his experimental conditions following challenge with the same tumor (but see ⁶), while removal of transplanted tumors recently induced with carcinogens consistently was found to result in immunity against subsequent challenge with the same tumor.

⁹ L. A. ZIL'BER, *Uspekhi Sovremennoi Biologii* 30, 188 (1950). Translated at the National Institutes of Health.

¹⁰ H. M. HIRSCH, J. J. BITTNER, H. COLE, and I. IVERSEN, *Cancer Res.* 18, 344 (1958). — T. S. HAUSCHKA, *Cancer Res.* 12, 615 (1952). — A. GOLDFEDER, *Brit. J. Cancer* 8, 320 (1954). — E. J. FOLEY, *Cancer Res.* 13, 578, 835 (1953).

¹¹ C. HACKMANN, *Z. Krebsforsch.* 57, 164 (1950). — F. W. STEWART, *Texas Rep. Biol. Med.* 10, 239 (1952).

¹² T. S. HAUSCHKA, *Cancer Res.* 12, 615 (1952).

¹³ H. J. C. LUND, *Brit. J. Cancer* 11, 475 (1957).

¹⁴ T. S. HAUSCHKA, B. J. KVEDAR, S. T. GRINNELL, and D. B. AMOS, *Ann. N. Y. Acad. Sci.* 63, 683 (1956).

¹⁵ L. GROSS, *Cancer Res.* 3, 770 (1943).

¹⁶ E. J. FOLEY, *Cancer Res.* 13, 578, 835 (1953).

FOLEY's work has recently been confirmed by BALDWIN¹⁷ and by PREHN and MAIN¹⁸. These data are well accounted for on the basis that the carcinogen-induced tumors have undergone more mutational changes than the spontaneous tumors¹⁹ and are thus immunogenetically further removed from the host tissue than are spontaneous tumors. Antigens not present in normal host tissues have been reported in carcinogen-induced tumors by ZIL'BER *et al.*²⁰, NARTISSOV and ZIL'BER²¹, and WEILER²².

If a mutated tumor, regardless of its mode of origin, represents a problem in homograft immunity rather than isoimmunity with respect to the reaction elicited following implantation in the original host strain, why is it that such tumors frequently grow well in their hosts of origin? The answer to this apparent contradiction is this: the mutated tumor grows in its strain of origin, and the so-called 'market' tumors even grow easily in any number of strains despite the fact that they have become quite different immunogenetically from their hosts because, during the long-extended periods of transfer usually characteristic of such tumors, cell lines have been selected out which have very great invasive power and growth potential and which are thus able to establish themselves before the immune response of the host comes into play. That this accords with the facts is borne out by observations that it actually is easy to immunize against these tumors by appropriate methods which prevent the initial overgrowth of the host by the tumor²³; this explanation avoids ascribing to these tumors such attributes as 'reduced specificity' or 'antigenic simplification'.

MARTINEZ *et al.*²⁴ have recently described conditions under which certain mice proved immune to reinoculation of a transplantable tumor although they were already succumbing to lung metastases formed from the originally inoculated tumor. This phenomenon can be explained on a similar basis; i.e., the metastases were able to establish themselves at a time when no immunity had yet been produced and when, in fact, conditions of enhancement were present. A later reinoculation of tumor was unsuccessful due to establishment of immunity, although this immunity was not

¹⁷ R. W. BALDWIN, *Brit. J. Cancer* 9, 652 (1955).

¹⁸ R. T. PREHN and J. M. MAIN, *J. nat. Cancer Inst.* 18, 769 (1957).

¹⁹ L. C. STRONG, *Genetics* 11, 294 (1926); *Brit. J. Cancer* 3, 97 (1949).

²⁰ L. ZIL'BER, N. NARTISSOV, T. RIVKIND, and Z. BAIDAKOVA, *Vest. A.M.N. SSSR.* 3, 36 (1948). — L. ZIL'BER, V. FREIMAN, I. ZBARSKII, and S. DEBOV, *Dokl. Akad. Nauk SSSR.* 65, 97 (1949).

²¹ N. NARTISSOV and L. ZIL'BER, *Dokl. Akad. Nauk SSSR.* 65, 229 (1949).

²² E. WEILER, *Z. Naturforsch.* 11b, 31 (1956).

²³ E. J. FOLEY, *Cancer Res.* 13, 578 (1953). — H. J. C. LUND, *Brit. J. Cancer* 11, 475 (1957). — C. HACKMANN, *Z. Krebsforsch.* 50, 352 (1940). — L. SACHS and R. GALLILY, *J. nat. Cancer Inst.* 16, 1083 (1956). — M. K. BARRETT, *Origins of resistance to toxic agents* (Academic Press, Inc., New York 1955), p. 308. — H. C. STOERCK, T. BUDZILOVICH, and T. C. BIELINSKI, *J. Mt. Sinai Hosp.* 19, 169 (1952).

²⁴ C. MARTINEZ, J. B. AUST, and J. J. BITTNER, *Cancer Res.* 16, 1023 (1956).

sufficient, as has frequently been shown before, to interfere with already established tumor growth.

Exactly the same holds true of tumors that have arisen from normal tissues of the mouse *in vitro* in tissue culture. SANFORD *et al.*²⁵, in appraising the antigenic changes that may occur during this process, have shown that certain cell lines, in contrast to others, can establish tumors in the host precisely because their growth rate is such that they can keep ahead of the host's immune response.

It does not seem impossible, in view of these considerations, that tumor cells originate in the body by mutation or otherwise *all the time*, but that those most removed from the host tissue are destroyed by the body's immunological defense mechanisms; only those tumor cells which are *very similar* to the host tissue thus would have a chance to develop into a tumor because the immune response of the host directed against such cells would be very weak. At variance with this is the finding, referred to above, that carcinogen-induced tumors are immunogenetically further removed from the host than are spontaneous tumors. The question can be asked: If such tumors are antigenically different from their hosts, how can they establish themselves in the first place? The answer may well lie in the finding that a general decline in antibody production occurs soon after the application of carcinogens²⁶.

The possibility cannot, therefore, be excluded that the cancerous state may be accompanied by a lessened plasticity or lowered response of the reticulo-endothelial system. Carcinogenic and mutagenic agents, such as ionizing radiations, radiomimetic chemicals, and several carcinogenic substances are known to lower immune responsiveness; their use thus might result in both the induction of tumor cells as well as in increased opportunities for such cell variants to establish themselves. The demonstration of a reduced homograft response in cancer patients²⁷ and of anergic phenomena and altered skin homograft rejections in patients with Hodgkin's disease²⁸ lends some support to the possibility that this may be a factor also in some spontaneous cancers.

One piece of evidence in support of the hypothesis of spontaneous tumor formation proposed comes from the well-established fact²⁹ that normal tissue kept in tissue culture, where it escapes the immunological and other restraining influences of normal body control, frequently undergoes cancerous changes. Of course,

other causes or trigger mechanisms are by no means excluded for this effect to take place. That such tumorous tissues are, however, different from true spontaneous tumors is shown by the observation of SALK and WARD³⁰ (see also²⁵) that on reimplantation of the tissue-culture-derived tumors into the host of origin, cytotoxic antibodies against the tumors are formed; this has so far not been convincingly demonstrated with tumors of spontaneous origin. In this respect, tumors that have arisen *in vitro* resemble carcinogen-induced tumors which also, in contrast to spontaneous tumors, serve as good immunizing agents in the host of origin³¹. The conclusion seems inescapable that both the '*in vitro* tumors' and the carcinogen-induced tumors are immunogenetically further removed from the host than are tumors of spontaneous origin. In this connection it should be noted that recent experiments demonstrating successful immunization of animals against homologous, strainspecific tumors have, without exception, been based on work employing long-transplanted tumors, carcinogen-induced tumors, hybrid though compatible recipient hosts, or a combination of these³².

In view of this it would seem that the time is at hand to use, in the study of problems of tumor isoimmunity, spontaneous tumors rather than long-transplanted tumors, tissue culture-derived tumors, or carcinogen-induced tumors. McDOWELL³³ has pointed out that 'long-established transplanted tumors offer opportunity for analytical experimentation to discover mechanisms working in these tissues and in their hosts; knowledge of these mechanisms underlying specific phenomena may have general implications. However, in searching for specific means of inducing resistance to cells of the type found in spontaneous cases, cells of this type should be used in the tests'. These remarks are as apropos today as they were 20 years ago. They apply both to the type of cell used to induce immunity and to the type of tumor to be immunized against.

Zusammenfassung

Einige neue Ergebnisse auf dem Gebiete der Tumor-Immunität werden mit der Literatur in Einklang gebracht, und eine Hypothese zur spontanen Tumorbildung wird vorgeschlagen. Ferner wird auf die Notwendigkeit eingegangen, bei Tumor-Isoimmunitäts-Experimenten Spontanumtoren zu verwenden an Stelle von lange transplantierten, von *in vitro* entstandenen oder durch Karzinogene erzeugten Tumoren.

³⁰ J. E. SALK and E. N. WARD, *Science* **126**, 1338 (1957).

³¹ E. J. FOLEY, *Cancer Res.* **13**, 835 (1953). — R. W. BALDWIN, *Brit. J. Cancer* **9**, 652 (1955). — R. T. PREHN and J. M. MAIN, *J. nat. Cancer Inst.* **18**, 769 (1957). — L. GROSS, *Cancer Res.* **3**, 326 (1943); *J. Immunol.* **50**, 91 (1945).

³² H. J. C. LUND, *Brit. J. Cancer* **11**, 475 (1957). — C. MARTINEZ, J. B. AUST, and J. J. BITTNER, *Cancer Res.* **16**, 1023 (1956). — C. MARTINEZ, J. B. AUST, J. J. BITTNER, and R. A. GOOD, *Cancer Res.* **17**, 205 (1957). — M. A. FINK, G. D. SNELL, and D. KELTON, *Cancer Res.* **13**, 666 (1953). — M. A. FINK, P. SMITH, and M. V. ROTHLAUF, *Proc. Soc. exp. Biol. Med.* **90**, 590 (1955). — J. E. PRINCE, J. C. FARDON, L. G. NUTINI, and G. S. SPERTI, *Cancer Res.* **17**, 312 (1957).

³³ E. C. McDOWELL, J. S. POTTER, and M. J. TAYLOR, *Proc. nat. Acad. Sci., Wash.* **25**, 416 (1939).

²⁵ K. K. SANFORD, R. M. MERWIN, G. L. HOBBS, M. C. FIORAMONTI, and W. R. EARLE, *J. nat. Cancer Inst.* **20**, 121 (1958).

²⁶ C. HOCH-LIGETI, *Brit. J. exper. Pathol.* **22**, 233 (1941). — R. A. MALMGREN and B. E. BENNISON, *Cancer Res.* **12**, 280 (1952). — R. A. MALMGREN, B. E. BENNISON, and T. W. MCKINLEY, JR., *Proc. Soc. exp. Biol. Med.* **79**, 484 (1952). — I. DAVIDSOHN, K. STERN, and L. SABET, *Proc. Amer. Assoc. Cancer Res.* **2**, 102 (1956).

²⁷ C. M. SOUTHAM, A. E. MOORE, and C. P. RHOADS, *Science* **125**, 158 (1957).

²⁸ W. D. KELLY, R. A. GOOD, and R. L. VARCO, *Surg. Gynecol. Obstetr.* Submitted for publication.

²⁹ W. R. EARLE, *J. nat. Cancer Inst.* **4**, 165 (1943).

Brèves communications - Kurze Mitteilungen Brevi comunicazioni - Brief Reports

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Photochemical Initiation of Polymerization of Tetrafluoroethylene by Mercury Bromides

Although tetrafluoroethylene can usually be stored indefinitely in glass vessels, polymerization on surfaces was observed in some vessels attached to mercury manometers. Bromine had been used in part of the apparatus and it was thought that the polymerization followed slight contamination of the mercury with mercury bromide. Polymerization did not occur when the vessels were shielded from light. These observations indicated that mercury bromide might initiate photochemically a polymerization of tetrafluoroethylene. Experiments to test this theory were performed.

Equal portions of pure tetrafluoroethylene¹ were condensed into cleaned glass tubes, volume about 6 cm³, containing various materials and the tubes were sealed under vacuum. Except when the additive included mercury, the tetrafluoroethylene was freed from mercury by passage through a trap at -130°C. The pressure of the gas in the tubes was about 25 cm and the additives used, separately, were redistilled mercury, mercury treated with bromine vapour to form mercurous bromide with mercury in excess, mercuric bromide crystals, and dibromotetrafluoroethylene. One set of tubes was exposed in the laboratory and a duplicate set was kept in the dark.

No polymerization took place in tubes containing pure tetrafluoroethylene, or in the tubes kept in the dark, with the exception of that containing dibromotetrafluoroethylene which showed traces of polymer after some weeks. In the light, obvious polymerization was produced by mercurous bromide in three days and by mercuric bromide in a week. Polymerization caused by dibromotetrafluoroethylene was greater in the light than in the dark but still very slow. Mercury in the light produced traces of polymer dust after exposure for some months. Further observations were made with filtered light from an ME/D 250 watt mercury lamp. No polymerization was produced by 4358 Å light. With 3650 Å light both mercuric and mercurous bromide produced obvious polymerization in 24 h, mercurous bromide being more effective. Mercurous bromide solid has an orange fluorescence in 3650 Å light. Polymer formed in the presence of mercury bromides was attached either to the mercury bromide surfaces or to the glass surfaces and was not deposited as a dust from the gas phase. It was concluded that polymerization initiated by mercury bromides in room light is caused by the near ultra-violet light present. Contamination of tetrafluoroethylene with dibromotetrafluoroethylene reduces its stability even in the absence of light, but the effect is apparent only over a long period of time.

DOYLE² has observed that the absorption threshold of mercuric bromide solid is in the near ultra-violet and the above results show that this is also true for mercurous bromide. Light in the far ultra-violet causes dissociation of mercuric bromide vapour into HgBr and Br with one of the products excited³. The energy required for dissocia-

tion into unexcited products is 62 kcal per mole, which is less than the energy provided by 3650 Å light, 78 kcal, but dissociation of the excited state in this manner may be forbidden. The fluorescence observed with mercurous bromide solid is at longer wavelengths than that of HgBr in the vapour observed by WIELAND³. There may be an excited state of Hg₂Br₂ about 50 kcal above the ground state. This discussion gives no grounds for assuming that absorption by solid mercury bromides at 3650 Å leads to the formation of bromine atoms. Although a free radical polymerisation of tetrafluoroethylene is produced, this again does not prove that free gaseous bromine atoms are formed. The reaction is obviously heterogeneous and direct transfer of bromine atoms from the solid to adsorbed tetrafluoroethylene may take place.

B. ATKINSON

Department of Chemistry, Imperial College of Science and Technology, London, May 12, 1958.

Zusammenfassung

Eine heterogene Polymerisation von Tetrafluoräthylen wurde durch Merkuri- und Merkurobromide in natürlichem Licht oder solchem von 3650 Å Wellenlänge, nicht aber in blauem Licht, ausgelöst. Es ist möglich, dass Bromatome vom angeregten Quecksilberbromid zum adsorbierten Tetrafluoräthylen übertragen werden.

Untersuchungen über Aktinpolymerisation

Der quergestreifte Muskel enthält ungefähr 0,2–0,3% ATP, das vielleicht bei der Kontraktion eine wichtige Rolle spielt. Auch ist bekannt, dass die Struktur-Proteine des Muskels ATP enthalten oder durch ATP stark beeinflusst werden.

Weiter wissen wir, dass das Aktin ungefähr 1% gebundenes ATP enthält. Dieses ATP wird während der Polymerisation gespalten. STRAUB¹ meinte, dass Polymerisation und Spaltung eng zusammenhängen und dass die Spaltung kein enzymatischer Prozess ist. Dieser Zusammenhang wurde von mehreren Forschern angenommen (LAKI *et al.*, BÍRÓ, TURBA *et al.*, MOMMAERTS, BÁRÁNY *et al.*), von anderen abgelehnt (DUBUISSON, SNELLMAN und GELOTTE).

Im Aktin zerfällt während der Polymerisation das ATP durch einen unbekannten Mechanismus in ADP und anorganischen Phosphor. Nach der Spaltung wird anorganischer Phosphor an das Aktin gebunden (MORALES *et al.*²), während das ADP an demselben gebunden bleibt (FEUER *et al.*³). Wir wissen jedoch bis heute noch nicht, welche

¹ F. B. STRAUB und G. FEUER, *Biochim. biophys. Acta* 4, 455 (1950).

² M. F. MORALES, K. LAKI, J. GERGELY und L. P. CECCHINI, *J. cell. comp. Physiol.* 37, 477 (1950); zitiert M. DUBUISSON, *Muscular Contraction* (Springfield 1954), p. 77.

³ G. FEUER und M. WOLLEMAN, *Acta physiol. Hung.* 3, 267 (1952).

¹ B. ATKINSON, *J. chem. Soc.* 1952, 2684.

² W. P. DOYLE, Thesis, University of London, 1951.

³ K. WIELAND, *Z. Phys.* 77, 157 (1932).

Rolle das ATP im Aktin *in vitro* und *in vivo* eigentlich spielt.

Zum Studium dieses Problems versuchten wir ein Aktin zu gewinnen, in dem das ATP während der Polymerisation nicht gespalten wird. Als Untersuchungsmaterial verwendeten wir Aktin, nach STRAUB⁴ zubereitet und nach GUBA modifiziert, sowie Kartoffel-Apyrase (nach SZÉKÉLY⁵). Die Untersuchungen mit ionenaustauschendem Harz wurden nach der Methode von HURLBERT *et al.*⁶, die ATP-Messungen (viskosimetrisch) nach KRAMER *et al.*⁷ ausgeführt. Zur Kontrolle der ATP-Bestimmung wurde nach der Methode CRANE und LIPMANN⁸ vorgegangen.

Um zu entscheiden, ob die ATP-Spaltung ein enzymatischer Prozess ist oder nicht, versuchten wir, diese Spaltung mit einem Hemmungsmittel während der Polymerisation beim gewöhnlichen Aktin zu verhindern.

Tabelle I

Viskositätsänderung in spezifischer Viskosität			Trockengewicht mg/ml
Aktin-Art	Glob.	Fibr.	
Gewöhnliches Aktin	0,12	1,0	3,0
Soxh.-Aktin	0,15	0,92	2,6

Tabelle II

Aktomyosin Komplex-Bildung	%
Gewöhnliches Aktin	119
Soxh.-Aktin	118

(Mittelwerte von vier Experimenten)

Wir polymerisierten das Aktin mit einer Salzlösung (1 m NaF 0,01 m MgCl₂). Nach Ausfällung des Eiweisses chromatographierten wir die filtrierte Lösung mit Dovex 2 nach der Methode HURLBERT. Es wurde kein ATP-Maximum beobachtet, was bedeutet, dass NaF unter diesen Umständen auf die Spaltung keinen Einfluss ausübte.

STRAUB und ULLMAN konnten 1953 durch Entfetten mit siedendem Azeton im Soxhlet-Apparat ein Aktin erzeugen, in dem das ATP während der Polymerisation nicht regelmässig gespalten wurde. Später gelang es uns jedoch, durch Modifizierung der Darstellungsmethode (Entfettung 1 h, 10 Reflux) nicht oder nur in geringem Mass spaltendes Aktin reproduzierbar herzustellen. Diese geringe Spaltung entsprach in keiner Weise dem Mol-ATP/Mol-Eiweissverhältnis (MOMMAERTS⁹). Dieses Aktin polymerisierte und vermochte Myosin zu binden (siehe Tabellen I und II). (Wir nennen dieses soxhletisierte Aktin weiter S-Aktin.)

Von 60 Aktin-Präparaten konnten wir in 10% eine Spaltung (die wohl dem Mol-ATP/Mol-Eiweissverhältnis entsprach) beobachten. In 50% geschah keine Spaltung, in 40% war die Spaltung bedeutend geringer als das Mol-ATP/Mol-Eiweissverhältnis.

⁴ F. B. STRAUB, *Studies on Muscle*, vol. 1 und 2 (1942).
⁵ M. SZÉKÉLY, *Acta physiol. Hung.* 1, 325 (1950).
⁶ R. B. HURLBERT, H. SCHMITZ, A. F. BRUMM und V. R. POTTER, *J. biol. Chem.* 209, 23 (1954).
⁷ M. KRAMER, E. PETKÓ und F. B. STRAUB, *Kisér. Orv. Tud. I.* 114 (1949).
⁸ R. K. CRANE und F. LIPMANN, *J. biol. Chem.* 201, 235 (1953).
⁹ W. F. H. M. MOMMAERTS, *Phosphorus Metabolism*, vol. 1 (The Johns Hopkins Press, Baltimore 1951), p. 551.

Weiter wurde von uns die ATP-Eiweissbindung im Verlauf der Extraktion untersucht. Zu diesem Zwecke wählten wir eine Apyrase-Konzentration, die den gebundenen ATP-Gehalt einer gewöhnlichen G-Aktinlösung während 30 min bei Zimmertemperatur nicht spalten konnte, wohl aber das freie ATP. Anschliessend wurde das Aktin statt mit destilliertem Wasser mit einer solchen Apyrase-Lösung während 20 min bei Zimmertemperatur aus trockenem Muskelpulver extrahiert. Die Resultate zeigen, dass im Mittelwert mindestens 70% des ATP-Gehaltes vom Extraktionsbeginn an nicht spaltbar an Eiweiss gebunden sind, oder dann maskiert.

Nun untersuchten wir, wie stark das ATP im S-Aktin gebunden ist im Vergleich zu gewöhnlichem Aktin. Mit Apyrase-Lösung verschiedener Konzentration inkubierten wir das gewöhnliche und das S-Aktin während 30 min bei Zimmertemperatur und bestimmten das ATP viskosimetrisch. Hierbei konnte festgestellt werden, dass das ATP im gewöhnlichen G-Aktin stärker gebunden ist als im globulären S-Aktin und im letzteren stärker als im polymerisierten (fibrillären) S-Aktin. Im fibrillären S-Aktin kam es öfters vor, dass das ATP ganz frei war.

Nach Angaben der Literatur ist das ATP und/oder ADP im Aktin vielleicht am Adeninkern, sogar an dem endständigen Phosphat gebunden (FEUER³, HASSELBACH¹⁰). Im S-Aktin ist diese Struktur von vornherein so verändert, dass die Bindungen frei oder geschwächt sind; hierbei spielen vielleicht die Fette und Lipide eine Rolle.

Die oben beschriebenen Experimente machen es wahrscheinlich, dass Polymerisation und ATP-Spaltung im Aktin zwar parallel verlaufen, aber doch voneinander unabhängig sind.

D. PRÁGAY*

Medizinisch-Chemisches Institut der Medizinischen Universität Budapest, 16. April 1958.

Summary

The suggestion is put forward that the polymerisation and ATP-splitting in actin are independent processes and that perhaps fats and lipoids are concerned in ATP-actin binding. (A comparison is made between the strength of ATP-binding of different actins.)

¹⁰ W. HASSELBACH, *Biochim. biophys. Acta* 25, 562 (1957).
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Sintesi e attività biologica
del 5-ammino-triptofano

L'interesse per lo studio dei derivati del triptofano si è fatto molto vivo in questi ultimi anni a causa sia dell'importanza del metabolismo di questo amminoacido, sia dell'effetto della serotonina e dei derivati indolici in generale sul sistema nervoso centrale. Ciò è stato causa della sintesi di parecchi derivati del triptofano recanti i più svariati sostituenti al nucleo benzenico.

In questi ultimi tempi abbiamo sintetizzato nei nostri Laboratori il 5-nitro-triptofano¹: per idrogenazione ca-

¹ G. CAVALLINI e F. RAVENNA, *Il Farmaco* (Ed. Sci.) 13, 105 (1958).

talitica di questo ho ora preparato il 5-ammino-triptofano che è, per quanto a mia conoscenza, il primo derivato del triptofano recante un gruppo amminico al nucleo benzenico. Questo prodotto presenta un certo interesse dal punto di vista teorico se si tiene conto del fatto che il 5-ossi-triptofano conserva ancora, sia pure in debole misura, l'attività vasocostrittrice della serotonina². Poichè le attività vasocostrittrici della 5-ammino- e della 5-nitro-triptamina sono rispettivamente uguali a circa $\frac{1}{3}$ e $\frac{1}{25}$ di quelle della serotonina³ mi è sembrato interessante studiare l'eventuale parallelismo fra l'attività biologica di un triptofano e della ammina corrispondente.

Le prove biologiche preliminari eseguite nei nostri Laboratori da MILLA e GRUMELLI, non hanno però dimostrato tanto per il 5-nitro- che per il 5-ammino-triptofano, fino ad una concentrazione di 100 μ /ml di liquido del bagno (Tyrode), alcun effetto contrattile sull'utero di ratto in estro; queste sostanze non hanno neppure esercitato, nelle medesime concentrazioni, alcun effetto inibitore della contrazione massimale dell'utero provocata da 0,1 μ /ml di serotonina (contrazione invece totalmente inibita da 0,1 μ /ml di Dibenamina).

Il dicloridrato del 5-ammino-triptofano è stato preparato nel modo seguente: 1 g di cloridrato di 5-nitro-triptofano¹ viene sciolto in 150 cm³ di etanolo al 90% e idrogenato a pressione ordinaria in presenza di 150 mg di biossido di platino secondo ADAMS. Il consumo di idrogeno corrisponde al teorico. Dopo filtrazione del catalizzatore e evaporazione del solvente sotto pressione ridotta in corrente di azoto, si sospende il residuo (0,9 g) a freddo in 3 cm³ di etanolo anidro saturo di HCl. Si filtra il dicloridrato così ottenuto e lo si lava 2-3 volte con delle piccole quantità di alcool isopropilico anidro. Il residuo così ottenuto (0,7 g) viene sospeso in 60 cm³ di alcool isopropilico e sciolto all'ebollizione aggiungendo il quantitativo minimo di acqua. Dopo filtrazione con carbone animale si raffredda e si aggiunge etere anidro fino a inizio di intorbidamento. Dopo alcuni giorni in ghiacciaia si filtrano i piccoli prismi incolori così ottenuti e li si lava con etere.

Resa 0,3 g. P.F. 278°C con dec. Trov.: % C 45,30; % H 5,29; % N 14,10 (per C₁₁H₁₃N₃O₂ · 2HCl calc.: % C 45,21; % H 5,17; % N 14,38).

R_f: 0,17 (acqua, *n*-butanolo, acido acetico 50:40:10). Macchia violacea con la ninidrina e aranciata con la dimetilaminobenzaldeide.

Assorbimento nell'U.V. max. 2245 (log ϵ 4,49)
2825 (log ϵ 2,66)
min. 2480 (log ϵ 3,03).

F. RAVENNA

Laboratori Ricerche Maggioni e C., Milano, 12 maggio 1958.

Summary

5-amino-tryptophan has been prepared by catalytic hydrogenation of 5-nitro-tryptophan.

These two products have been shown to be devoid of both serotonin-like and serotonin-inhibiting activity on uterus of rats in oestrus.

² A. EK e B. WITKOP, J. Amer. chem. Soc. 75, 500 (1953).

³ D. W. WOOLLEY e E. SHAW, J. biol. Chem. 203, 69 (1953).

Über Fritillari cyanin, ein neues Anthocyanin aus den Blüten der japanischen Schachblume

Die Blütenfarbstoffe der japanischen Schachblume, *Fritillaria camschatcensis* Ker-Gawl, wurden von K. HAYASHI *et al.*¹ papierchromatographisch untersucht; sie fanden zwei Anthocyane, nämlich Keracyanin (?) und Chrysanthemin.

Im Winter 1955 gelang es mir, den Blütenfarbstoff dieser Pflanze kristallisiert zu gewinnen. Das zweimal umkristallisierte luftgetrocknete Präparat zersetzte sich bei 182°C (unkorr.) unter Gasentwicklung und ergab die Formel C₂₆H₂₉O₁₄Cl · 3 H₂O. Im Hydrolysat wurde keine esterartig gebundene organische Säure, sondern nur drei Spaltstücke, das Aglykon und zwei Zucker, gefunden; das Aglykon war mit Cyanidinchlorid identisch; die Zucker erwiesen sich nach Farben-Reaktionen und Papierchromatographie als Xylose und Rhamnose. Nach spektrophotometrischen Untersuchungen scheint Farbstoff als Cyanidin-3-biosid vorzuliegen; Reihenfolge und Bindungsart der beiden Monosen im Disaccharid des ursprünglichen Farbstoffs sind noch ungewiss.

Diesem neuen Anthocyanin möchte ich die Bezeichnung Fritillari cyanin geben.

Eine ausführliche Mitteilung wird an anderer Stelle erfolgen.

M. SHIBATA

Pflanzenphysiologisches Laboratorium, Biologisches Institut der Universität Toyama (Japan), 8. April 1958.

Summary

Fritillari cyanin, the colouring principle of the dark purple flower of *Fritillaria camschatcensis* Ker-Gawl, has been crystallized. It is a cyanidin xylorhamnoside.

¹ K. HAYASHI und Y. ABE, Bot. Mag. Tokyo 69, 227 (1956).

Le rôle de l'hétérosis dans la compétition entre *ebony* et son allèle normal

L'observation directe du comportement sexuel a montré que la mutation *ebony* est responsable, à l'état homozygote, d'une moindre activité des mâles. Nous émettions l'hypothèse, dans une note précédente¹, que l'avantage hétérotique auquel on attribue parfois le maintien du gène *ebony* dans les populations mixtes pourrait consister partiellement en une plus grande activité des hétérozygotes, ce que certaines observations semblaient suggérer. Voici quelques précisions à ce sujet.

Matériel et techniques. Nous utilisons les souches *ee(e)* et ++(+) à cytoplasme *ebony e*¹¹, *ee(+)* et ++(+) à cytoplasme *sauvage* Canton spécial obtenues par une méthode que nous avons déjà décrite. Pour l'expérimentation, nous employons cette fois des cages circulaires, d'un diamètre intérieur de 10 cm environ (elles étaient fabriquées par F. SCHOLLAERT, technicien au Laboratoire de Technologie Forestière de l'Institut Agronomique). Les individus utilisés étaient âgés de 4 à 5 jours. Les mesures d'activité portaient sur 50 couples vierges. Pour les tests d'isolation sexuelle, il était nécessaire de distinguer les hétérozygotes, qui phénotypiquement ne diffèrent pas

¹ A. A. ELEN, Exper. 13, 293 (1957).

des *sauvages*. Ceux de père *sauvage* – + *e* – étaient marqués d’une tache verte, ceux de père *ebony* – + – d’une tache rouge au thorax, en se servant des «magic markers» des «Speedry Products» de Richmond Hill, N.Y., USA. Des expériences préalables avaient en effet montré que les accouplements se font au hasard entre mouches *sauvages* marquées de rouge et de vert, et que l’activité générale n’est pas affectée par le marquage. Dans l’avenir cependant, une couleur autre que le rouge serait à préférer, qui tranche mieux sur la couleur normale du thorax. Chaque mesure portait sur 25 couples vierges des quatre catégories *sauvages* (++) , *ebony*(*ee*), hétérozygotes de père *sauvage* (+*e*), hétérozygotes de père *ebony*(*ee*), soit 100 couples au total.

Table I

	Cytoplasme (<i>e</i>)				Cytoplasme (+)			
	++	+ <i>e</i>	<i>e</i> +	<i>ee</i>	++	+ <i>e</i>	<i>e</i> +	<i>ee</i>
17°C	10	21	46	4	26	76	44	16
25°C	65	80	69	11	80	66	76	27
30°C	26	54	21	4	31	84	37	6

Résultats et discussion. La Table I relève le nombre de copulations observées pour 100 couples de chaque catégorie (décembre 1957), la Table II groupe les résultats de 8 tests de choix sélectif (mars 1958) portant au total sur 800 couples dans chacune des souches (+) et (*e*). Comme on pouvait s’y attendre d’après les expériences précédentes, les données de la Table II sont inconciliables avec l’hypothèse de la panmixie: les mâles *ee* se montrent bien moins actifs que les mâles ++, d’autre part il semble bien y avoir, en certains cas tout au moins, un réel avantage hétérotique. En présence du cytoplasme (*e*) les hétérozygotes de père *sauvage* se montrent nettement plus actifs que les ++ eux-mêmes, tandis que les produits du croisement réciproque sont moins actifs. Par contre, dans l’autre lignée (+) à cytoplasme *sauvage*, ce sont plutôt les hétérozygotes de père *ebony* qui font montre d’une activité légèrement plus grande. Les données de la Table I permettent, une fois de plus, d’attirer notre attention non seulement sur l’importance du sens du croisement, mais encore sur la relativité de la notion d’hétérosis en fonction des conditions physiques. A basse température les hétérozygotes – quelque soit le sens du croisement – sont toujours plus actifs que les *sauvages*, mais il n’en va pas nécessairement de même aux températures plus élevées. Il n’est pas sans intérêt de signaler ici qu’à 25°C, lors du croisement des deux souches originales *sauvage* Canton spécial et *ebony e*¹¹, nous avions jadis obtenu 76% de copulations pour les hétérozygotes de père *sauvage*, 62% pour ceux de père *ebony*, 60% pour les homozygotes *sauvage* et 20% pour les *ebony*, tandis qu’à 17°C et 30°C les hétérozygotes se montraient toujours plus actifs, quel que soit le sens du croisement (avril 1957). En septembre 1956, nous obtenions, à 25°C: 32% pour les *ebony*, 52% pour les *sauvages*, et 70% pour les deux hétérozygotes. Toutes ces données ne sont évidemment pas rigoureusement comparables, puisqu’il est évident que le niveau général d’activité varie au cours du temps: en septembre 1956, avril 1957, décembre 1957, mars 1958 nous trouvons

respectivement 72%, 56%, 77% et 42% pour les lignées à cytoplasme (*e*), 57%, 62%, 65% et 32% pour les lignées à cytoplasme (+). Les activités relatives peuvent aussi varier, ainsi que le montre la comparaison de la Table II avec les données de la Table I relatives à 25°C. Il serait donc souhaitable de pouvoir procéder à des mesures régulières de l’activité sexuelle pendant une durée assez longue.

Est-il possible à partir de ces résultats de tirer quelques conclusions relatives aux populations artificielles? Les conditions des cages où nous observons le comportement sexuel ne sont pas exactement celles des élevages, où l’activité sexuelle est loin de se restreindre à ce que nous en voyons pendant les deux premières heures. Cependant, même si l’activité des mâles *ebony* demeure constante ou même s’élève pendant les jours qui suivent la mise en route de l’élevage, la très grande supériorité des mâles *sauvage* pendant les deux premières heures pourrait contribuer à expliquer la diminution si rapide de la proportion d’*ebony* dès les premières générations.

Quant au maintien en équilibre du gène *ebony*, dans les générations suivantes, en quelle mesure peut-il dépendre d’une plus grande activité sexuelle des hétérozygotes? Certainement pas de façon exclusive, ainsi que L’HÉRITIER et TEISSIER l’ont montré depuis longtemps². La concurrence larvaire est aussi un facteur important. Cependant les résultats des expériences d’élevage sont assez en accord avec nos observations sur l’activité sexuelle. Pour des populations composées au départ de 20 couples vierges *ebony e*¹¹ et 20 couples vierges *sauvages* Canton spécial, la proportion d’*ebony* se stabilisa aux environs de 8% à 30°C, de 6% à 25°C, de 18% à 17°C. Or, d’après les tests d’activité sexuelle, c’est surtout à basse température, et aussi, dans une moindre mesure, à 30°C que se manifeste la supériorité des hétérozygotes, tandis qu’à 25°C, seuls ceux de père *sauvage* semblent avantagés. Ces élevages – selon la méthode de BUZZATI-TRAVERSO³ – concernaient les souches *e*¹¹ et Canton spécial. D’autres – selon la technique de REED⁴ – eurent comme point de départ les lignées à cytoplasme (*e*) et (+) sélectionnées après 12 générations de croisements répétés dans le même sens. A 30°C la population ne put se maintenir dans aucun cas. A 25°C la stabilisation se fit aux environs de 2% pour les lignées (*e*), de 4% pour les lignées (+); à 17°C vers 9% dans les deux cas. Contrairement à ce qu’on aurait pu attendre, les différences cytoplasmiques n’influencent donc guère, ou même pas du tout, le niveau d’équilibre. Ce qui signifie peut-être que ces différences, assez nettes, comme le montrent les tests d’activité sexuelle, après 30, ou même 12 générations de croisements répétés dans le même sens, disparaissent tout simplement lorsqu’*ebony* et *sauvages* se trouvent à nouveau réunis dans une même population.

A. A. ELENS

Laboratoire de Génétique générale, Institut Agronomique de l’Université de Louvain, le 23 avril 1958.

² Ph. L’HÉRITIER et G. TEISSIER, C. R. Soc. Biol. 124, 880 (1937).
³ A. BUZZATI-TRAVERSO, Mem. Ist. ital. Idrobiol. 4, 41 (1947).
⁴ S. REED in M. DEMEREC, *Biology of Drosophila* (Wiley & Sons, London 1950).

Table II

♀♀	♂ ++					♂ + <i>e</i>					♂ <i>e</i> +					♂ <i>ee</i>				
	++	+ <i>e</i>	<i>e</i> +	<i>ee</i>	Total	++	+ <i>e</i>	<i>e</i> +	<i>ee</i>	Total	++	+ <i>e</i>	<i>e</i> +	<i>ee</i>	Total	++	+ <i>e</i>	<i>e</i> +	<i>ee</i>	Total
Cytopl. (<i>e</i>)	30	27	16	19	92	44	50	35	24	153	25	24	19	16	84	6	2	2	2	12
Cytopl. (+)	22	22	18	21	83	10	26	16	23	75	14	37	18	26	95	0	1	2	1	4

Summary

Whereas in homozygous state the gene *ebony* noticeably diminishes the sexual activity of the males, it can on the contrary increase it in heterozygous state; but this heterotic effect depends in a large measure on the direction of the crossing, and also on the genic and cytoplasmic context. It depends moreover on the temperature.

The role of this activity in the populations where *ebony* is made to compete with its normal allele is discussed briefly.

Über den Intermediär-Stoffwechsel von *Aspergillus niger*

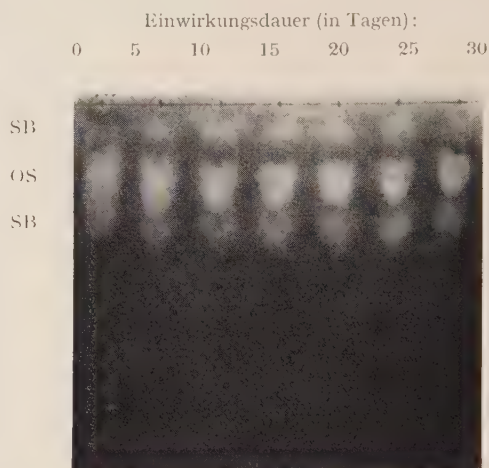
Aspergillus niger vermag alle Zwischenglieder des Tricarbonsäurezyklus zu metabolisieren, so dass bei ihm der Ablauf des KREBSSchen Abbauschemas als gesichert erscheint. Daneben sind aber noch andere Reaktionsmöglichkeiten in Erwägung zu ziehen.

So produziert der Pilz unter geeigneten, für das Wachstum wenig günstigen Bedingungen bekanntlich in erheblicher Menge Zitronensäure. Diese Anreicherung eines einzelnen Metaboliten muss offenbar als pathologisch, als Störung des normalen Ablaufes des KREBS-Zyklus gedeutet werden¹. Die Arbeiten von RAMAKRISHNAN *et al.*² haben in Verfolgung derartiger Überlegungen gezeigt, dass während des Gärablaufes die Aktivität des «condensing enzyme» eines zitronensäurereaktiven *A. niger*-Stammes ansteigt, diejenige der Aconitase und der Isocitricodehydrase dagegen erheblich absinkt, um schliesslich völlig zu verschwinden. Nach Verbrauch der dargebotenen Kohlenhydrate (zum Beispiel Glucose) ist der gleiche Organismus befähigt, die primär gebildete Zitronensäure wieder in den Stoffwechsel einzubeziehen; dies erfolgt – wie eigene sowie die Versuche anderer Autoren gezeigt haben³ – auch in Gegenwart des Aconitasehemmstoffes Fluoressigsäure.

Besondere Erwägung verdient weiterhin die Tatsache, dass der Schimmelpilz auf Acetat – wie auch in Gegenwart anderer C₂-Verbindungen – als einziger Kohlenstoffquelle zu wachsen bzw. diese Stoffe zu metabolisieren in der Lage ist⁴. Gemäss KORNBERG und COLLINS⁵ ist der von KORNBERG und KREBS⁶ nach Untersuchungen vor allem an *Pseudomonas* aufgestellte «Glyoxylsäurezyklus» offenbar auch für den Umsatz von Acetat durch *A. niger* verantwortlich zu machen. Die Synthese von Zellmaterial aus C₂-Verbindungen würde durch diese Reaktionsfolge (Acetat → Glyoxylsäurezyklus → Malat → umgekehrte Glykolyse → Kohlenhydrat) auf diese Weise eine plausible Erklärung finden.

In diesem Zusammenhang scheinen einige Ergebnisse unserer Untersuchungen zum Säureabbau verschiedener Säuren durch vorgebildete, entsprechend vorbereitete Myzeldecken von *A. niger* von Interesse zu sein. Oxalsäure wird zum Beispiel vom Myzel in geeigneter Konzentration (1%) nur in Gegenwart einer anderen Kohlenstoff-

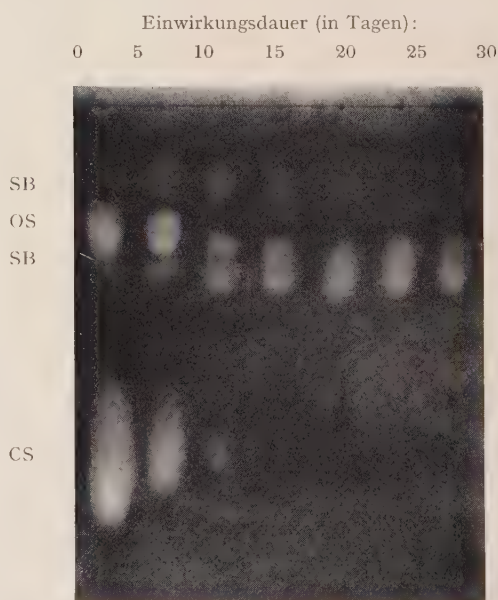
quelle (zum Beispiel Zitronensäure, Bernsteinsäure), nicht dagegen als Einzelkomponente umgesetzt (Abb. 1 und 2)⁷. Einen andersartigen Befund erhält man bei Verwendung von Malonsäure. Der Pilz vermag dieselbe so-



Erläuterungen. OS: Oxalsäure; SB: Saure Bestandteile (Salze) der Nährlösung

Abb. 1. Chromatogramm der sauren Bestandteile einer mit *A. niger* (Myzeldecke) bebrüteten Lösung der notwendigen Nährsalze unter Zusatz von 10⁻¹ Mol/l Oxalsäure

wohl in Gegenwart einer anderen Kohlenstoffquelle (zum Beispiel Zitronensäure, Bernsteinsäure) wie auch allein abzubauen (Abb. 3 und 4); im letzteren Falle erfolgt dies allerdings wesentlich verlangsamt.



Erläuterungen. OS: Oxalsäure; CS: Zitronensäure; SB: Saure Bestandteile (Salze) der Nährlösung

Abb. 2. Chromatogramm der sauren Bestandteile einer mit *A. niger* (Myzeldecke) bebrüteten Lösung der notwendigen Nährsalze unter Zusatz von 3% Zitronen- und 10⁻¹ Mol/l Oxalsäure

¹ Vgl. K. TÄUFEL und H. RUTTLOFF, Ernährungsforsch. 1, 271 (1956); Nahrung 1, 303 (1957).

² C. V. RAMAKRISHNAN, R. STEEL und C. P. LENTZ, Arch. Biochem. Biophys. 55, 270 (1955).

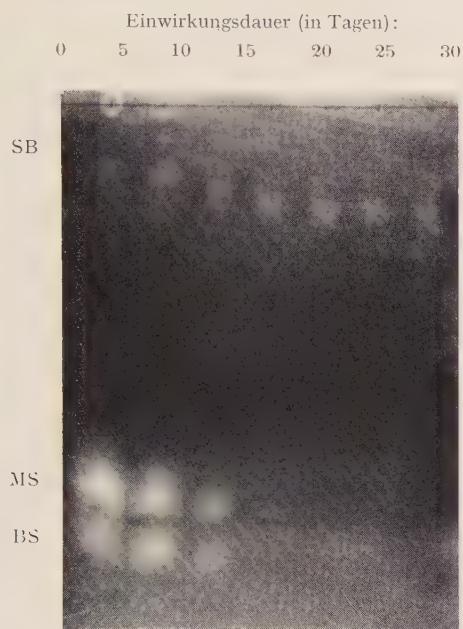
³ E. JENEY und T. ZSOLNAY, Zbl. Bakteriol. I, Orig. 168, 453 (1957).

⁴ K. BERNHAUER und Z. SCHEUER, Biochem. Z. 253, 11 (1932). – T. CHRZASZCZ und M. ZAKOMORNY, Biochem. Z. 285, 340 (1936).

⁵ H. L. KORNBERG und J. F. COLLINS, Biochem. J. 68, 3 P (1958).

⁶ H. L. KORNBERG und H. A. KREBS, Nature (London) 179, 988 (1957).

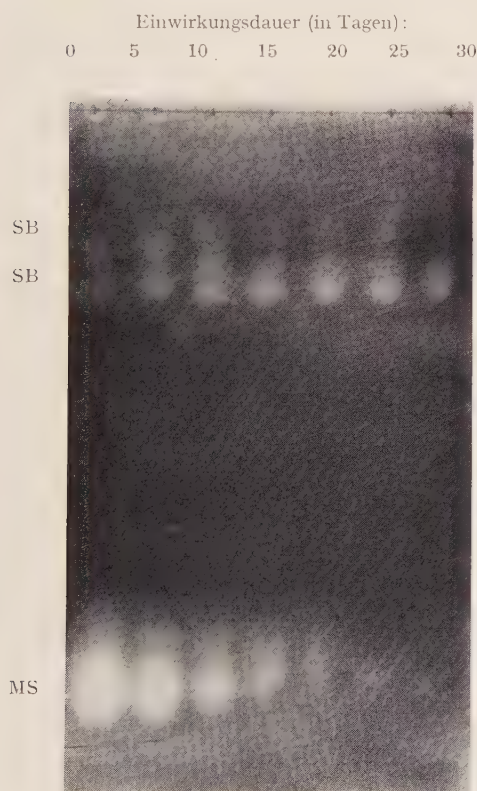
⁷ Vgl. A. ALLSOPP, J. exp. Bot. 1, 71 (1950). – H. MÜLLER, Arch. Mikrobiol. 15, 137 (1950).



Erläuterungen

SB: Saure Bestandteile (Salze) der Nährlösung; MS: Malonsäure;
BS: Bernsteinsäure

Abb. 3. Chromatogramm der sauren Bestandteile einer mit *A. niger* (Myzeldecke) bebrüteten Lösung der notwendigen Nährsalze unter Zusatz von 3% Bernstein- und $3 \cdot 10^{-1}$ Mol/l Malonsäure



Erläuterungen

MS: Malonsäure; SB: Saure Bestandteile (Salze) der Nährlösung.

Abb. 4. Chromatogramm der sauren Bestandteile einer mit *A. niger* (Myzeldecke) bebrüteten Lösung der notwendigen Nährsalze unter Zusatz von $3 \cdot 10^{-1}$ Mol/l Malonsäure

Aus diesen Ergebnissen ist zu folgern, dass Oxalsäure – als Lieferant von Kohlendioxyd und Wasser⁸ – lediglich in Gegenwart solcher Kohlenstoffquellen metabolisiert wird, die neben der Energielieferung zugleich einen Ersatz für die sich laufend abnutzende Zellschubstanz sicherstellen. Das entstehende Kohlendioxyd wird möglicherweise – wenigstens partiell – zur Carboxylierung von Intermediärgliedern (zum Beispiel zur Zitronensäureerzeugung) herangezogen⁹. Wenn man im Falle der Malonsäure eine Decarboxylierung zu Essigsäure unterstellt¹⁰, so sind – auch bei Zugabe der erstgenannten als einziger Kohlenstoffquelle – sowohl die Energiegewinnung (Zitronensäurezyklus) wie auch der Aufbau von Zellschubstanz (Glyoxylsäurezyklus) gewährleistet, das heisst das Myzel ist lebensfähig; über experimentelle Einzelheiten sowie detaillierte Ergebnisse wird an anderer Stelle ausführlich berichtet werden.

K. TÄUFEL und H. RUTTLÖFF

Deutsche Akademie der Wissenschaften zu Berlin, Institut für Ernährung in Potsdam-Rehbrücke, 23. Mai 1958.

Summary

Oxalic acid is only dissimilated if other carbon sources are present (e.g. citric acid, succinic acid), whereas malonic acid is also dissimilated as a single component by *A. niger*. It is supposed that the latter is used by the mould not only energetically but also for building up cell substance.

⁸ G. BRUBACHER, M. JUST, H. BODUR und K. BERNHARD, Hoppe-Seylers Z. physiol. Chem. 304, 173 (1956).

⁹ W. W. CLELAND und M. J. JOHNSON, J. biol. Chem. 208, 679 (1954). – Vgl. G. HALLIWELL, J. exp. Bot. 4, 369 (1953).

¹⁰ Vgl. O. HAYAISHI, J. biol. Chem. 215, 125 (1955).

Effects of Salicylate on Water and Electrolyte Content of Human Red Blood Cells

Changes in water and electrolyte content of patients treated with salicylate for acute rheumatic fever were examined by REID *et al.*¹. They concluded, from plasma and urine volume and electrolyte balance determinations, that during salicylate medication the body retains sodium and loses potassium, whilst the cellular water content decreases. The observations of KELEMEN and TANOS² on rats differ from the above-mentioned statements in one point only: In their *acute* salicylate experiments, the cellular fluid did not diminish, but increase. The addition of salicylate *in vitro* leads to a great loss of potassium from isolated rat diaphragm during incubation (MANCHESTER *et al.*³).

We demonstrated that 4 h after giving a high dose of sodium salicylate (6–10 g *per os*) to normal human subjects, the erythrocytes undergo the following alterations: (1) They lose 11.95 mEq/l cell potassium ($p < 0.001$). (2) They take up 4.22 mEq/l cell sodium ($0.01 > p > 0.001$) and 37 ml/l cell water ($p < 0.001$) with a simultaneous increase in their diameter of 0.46μ ($0.05 > p > 0.01$) (mean of 7 experiments)⁴.

¹ J. REID, R. D. WATSON, J. B. COCHRAN, and D. H. SPROULL, Brit. med. J. 1951, ii, 321.

² E. KELEMEN and B. TANOS, Acta med. hung. 11, 445 (1957).

³ K. L. MANCHESTER, P. J. RANDLE, and G. H. SMITH, Brit. med. J. 1958, i, 1028.

⁴ Serum salicylate levels ranged from 32 to 48 mg %.

Furthermore the *in vitro* effect of salicylate on human red blood cells were examined. The erythrocytes were incubated in plasma at 37°C in a shaking apparatus. Sodium salicylate was added in a 50 mg% concentration. At the 60th min, glucose was added to produce a 100 mg% increase in glucose level. Erythrocytes from the same person were similarly treated except for adding salicylate. After 2 h, the potassium, sodium and water content and the average diameter of the red blood cells in both systems were determined. After washing the cells 3 times in isotonic glucose, electrolyte measurements were made in the haemolysate using a flame photometer.

On the average (mean of 7 experiments), the salicylate-treated cells extrude 8.14 mEq/l cell potassium ($p > 0.001$), take up 1.69 mEq/l cell sodium ($p < 0.001$) and 45 ml/l cell water ($p < 0.001$), their diameter increasing 0.385 μ ($0.01 > p > 0.001$).

We interpret our findings as a form of the 'tired cell' syndrome described by ELKINTON⁵. Salicylate is known to uncouple oxidative phosphorylation in the cells. Another important consequence is, however, the development of an energy deficiency state (SMITH and JEFFREY⁶, KELEMEN and TANOS³). Owing to these alterations, the cells cannot maintain the normal concentration gradient against plasma, i. e. a netto potassium lost from the erythrocyte takes place under salicylate action. In this connection it may be mentioned that the insufficiency to maintain water and electrolyte balance in slices or mitochondrial preparations of hyperthyreotic animals—as manifested both in swelling and in potassium lost—was interpreted by AEBI and ABELIN⁷ on the base of the insufficiency of high energy phosphate bonds, produced by uncoupling of oxidative phosphorylation in the hyperthyreotic subject.

Changes in water and electrolyte content of human red blood cells produced by salicylate

	Difference in electrolyte content in mEq/l erythrocytes		Increase in water content in ml/l cells	Increase in erythrocyte diameter in μ
	K	Na		
<i>In vivo</i> . . .	− 11.95	+ 4.22	+ 37	+ 0.46
<i>In vitro</i> . . .	− 8.14	+ 1.69	+ 45	+ 0.385

The present observations suggest, that these effects of salicylate are due to a direct action on the cell requiring no mobilisation of endocrine or other mediating factors.

K. WALTNER, JR., M. CSERNOVSZKY, and E. KELEMEN

1st Department of Medicine, University Medical School, Szeged (Hungary), May 14, 1958.

Zusammenfassung

Es wird gezeigt, dass Natriumsalicylat *in vitro* auf menschliche Erythrozyten einen direkten Einfluss ausübt, indem es den Kaliumgehalt vermindert und den Natrium- sowie den Wassergehalt vermehrt. Diese Wirkung, die auch *in vivo* beobachtet werden kann, dürfte die Folge einer Energieverarmung sein, die durch eine Entkoppelung der oxydativen Phosphorylierung zustande kommt.

Beziehung zwischen Nebenniere und aktivem Kationentransport

Untersuchungen zur Abklärung der durch Herzglykoside hervorgerufenen Hemmung des aktiven Kationentransportes durch die Erythrozytenmembran haben gezeigt, dass diese Blockierung nicht auf einer energetischen Insuffizienz der Zelle im Sinne einer Abnahme energiereicher Phosphatverbindungen beruht, sondern dass ein direkter Angriffspunkt am Membrantransportmechanismus wahrscheinlich ist¹. Es wurde die Interpretation der Verdrängung eines Steroidchelats durch Herzglykoside vorgeschlagen und diese experimentell durch den Nachweis einer antagonistischen Beeinflussung der Hemmwirkung von k-Strophanthosid auf den aktiven Natriumtransport durch gewisse Corticosteroide am «Kälte-Erythrozyten»² und durch eine antagonistische Wirkung von Herzglykosid und Corticosteroid auf die renale Natriumausscheidung an der adrenaletomierten Ratte gestützt³. Ausgehend von der Annahme, dass «genuine» Corticosteroide Trägermoleküle für den aktiven Kationentransport darstellen könnten, wurde der Einfluss beidseitiger Adrenaletomie auf den aktiven Natrium-Kaliumtransport beim «Kälte-Erythrozyten» neben-nierenloser Ratten und die Hemmbarkeit des aktiven Kationentransportes durch Herzglykoside im Zustand der Insuffizienz geprüft.

Methodik. 120–140 g schwere männliche Albinoratten wurden durch Dekapitation getötet. Das ausfliessende Blut wurde durch Röhren mit Kunststoffstäbchen defibriniert, der Hämatokritwert bestimmt und während 4–6 Tagen bei 4°C aufbewahrt. Die nachfolgenden Untersuchungen über aktiven Transport erfolgten nach der früher angegebenen Methode⁴. Die totale Hämolyse wurde durch Zusatz von Digitonin erreicht. Die bilaterale Adrenaletomie erfolgte nach BURN⁵. Da akzessorische Nebennieren nach erfolgtem Eingriff relativ häufig zu beobachten sind, wurden nur Tiere in ausgesprochenem Insuffizienz-zustand (Gewichtsabnahme, Adynamie, hoher Hämatokritwert) für die Versuche verwendet.

Resultate. Vergleicht man die aktive Natriumverschiebung durch die Erythrozytenmembran normaler Ratten mit derjenigen insuffizienter adrenaletomierter Tiere, so ergibt sich für die letztere Gruppe eine statistisch hoch signifikante Verlangsamung dieses Austauschvorganges. Ein Beispiel zeigt Abbildung 1. Die mittlere Stundengeschwindigkeit des Natriumaustrittes in der ersten Stunde beträgt bei intakten Kontrolltieren in meq Natrium/l Zellen $9,89 \pm 0,90^6$ ($n = 13$), bei adrenaletomierten Tieren $2,98 \pm 0,28^6$ ($n = 11$) bei einer Zufallswahrscheinlichkeit des Unterschiedes von $P \ll 0,001$.

In Abbildung 2 ist die mittlere Stundengeschwindigkeit des aktiven Netto-Auswärtstransportes von Natrium, bzw. des Netto-Einwärtstransportes von Kalium bei Erythrozyten normaler und adrenaletomierter Tiere gegen den Hämatokritwert aufgetragen. Ist die Verschiebung des Hämatokritwertes ein Mass für den Insuffizienz-zustand adrenaletomierter Tiere, so geht die Verlang-

¹ H. A. KUNZ und F. SULSER, Exper. 13, 365 (1957). – R. WHIT-TAM, J. Physiol. 137, 13 P (1957).
² F. SULSER und W. WILBRANDT, Helv. physiol. Acta 15, C37 (1957).
³ R. GANTENBEIN, F. SULSER und W. WILBRANDT, Helv. physiol. Acta 15, C 64 (1957).
⁴ H. A. KUNZ und F. SULSER, Exper. 13, 365 (1957).
⁵ J. H. BURN, Biological Standardization (Oxford University Press, London 1950).
⁶ Mittlerer Fehler des Mittelwertes.

⁵ J. R. ELKINTON, Circulation 14, 1027 (1956).
⁶ M. J. H. SMITH and S. W. JEFFREY, Biochem. J. 64, 589 (1956).
⁷ H. AEBI and J. ABELIN, Biochem. Z. 324, 364 (1953).

samung des aktiven Natriumtransportes beim Kälteerythrozyten der Schwere der Insuffizienz parallel. Für den aktiven Kaliumtransport lässt sich diese Beziehung nicht nachweisen. Die mittlere Stundengeschwindigkeit des

wurde die Kaliumpumpe nicht beeinflusst, zumindest nicht in der ersten Stunde, wo noch keine Hämolyse die Bestimmung der gewanderten Kaliummenge erschwerte. (Eine ausführliche Darstellung dieser Entkoppelungsphänomene erfolgt an anderer Stelle⁷.)

Da die beobachteten Effekte Bilanzen zwischen aktivem Transport des Natriums entgegen dem elektrochemischen Gradienten und passiver Verschiebung entsprechend dem Konzentrationsgradienten darstellen, so wäre dasselbe Ergebnis denkbar nicht nur durch eine Hemmung des aktiven Transportes, sondern auch durch eine Beschleunigung des passiven Einstroms von Natrium. Orientierende Versuche zeigten, dass während der Kaltlagerung des Blutes hinsichtlich Geschwindigkeit der passiven Natriumeinwanderung in die Zellen keine Unterschiede zu bestehen scheinen zwischen Erythrozyten normaler und adrenaletomierter Tiere. Eine stärkere Hemmwirkung auf den aktiven Natriumtransport bei Erythrozyten nebennierenloser Tiere durch herzwirksame Glykoside, wie man sie hätte erwarten können, liess sich nicht eindeutig beurteilen, da nach Adrenaletomie allein schon eine sehr ausgeprägte Abnahme des aktiven Natriumaustrittes zu beobachten war. Hingegen fiel bei diesen Versuchen auf, dass k-Strophanthosid sowohl bei Erythrozyten normaler wie adrenaletomierter Tiere selektiv den aktiven Natriumtransport hemmt, während der aktive Kaliumeinstrom unbeeinflusst bleibt.

Die vorliegenden Untersuchungsergebnisse an Erythrozyten nebennierenloser Ratten bilden eine weitere Stütze für die Vorstellung, dass «genuine» Corticosteroide als Trägermoleküle für den aktiven Natriumtransport dienen könnten. Auch die Resultate andersartiger Versuche von WOODBURY und KOCH⁸ und von GROLLMAN⁹ könnten in gleicher Richtung interpretiert werden. Die von SCHATZMANN¹⁰ und SHERWOOD JONES¹¹ beobachtete Hemmwirkung gewisser Nebennierensteroiden in hoher Konzentration auf den aktiven Kationentransport *in vitro* beruhen möglicherweise darauf, dass unter bestimmten experimentellen Bedingungen nicht «genuine» Corticosteroide eine Verdrängung der physiologischen Corticoide und damit digitalisähnliche Effekte bewirken können. Entsprechende Befunde hinsichtlich renaler Elektrolytausscheidung bei der Ratte wurden in gleicher Weise gedeutet¹².

H. A. KUNZ und F. SULSER

Pharmakologisches Institut der Universität Bern, 24. Mai 1958.

Summary

The active sodium transport through the membrane of coldstored red cells of adrenalectomised rats is significantly decreased, whereas the rate of active exchange of potassium is not influenced by adrenalectomy. Cardiac glycosides likewise slow down only the active sodium exchange in the blood of both the normal and adrenalectomised animals in contrast to their action on human red cells.

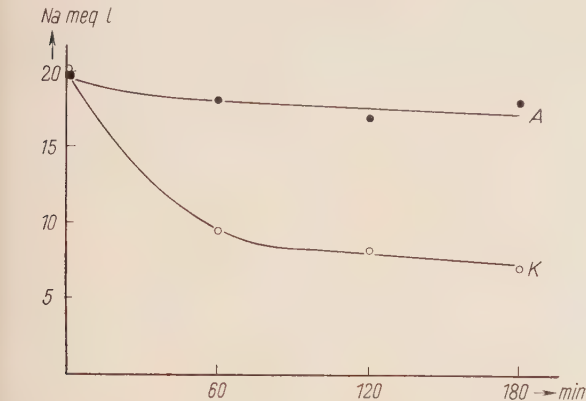


Abb. 1. Hemmung des aktiven Natriumtransportes nach Adrenaletomie. Ordinate: Natrium in meq/l Zellen. Abszisse: Inkubationszeit in Minuten. A = Blut einer adrenaletomierten Ratte, 6 Tage bei 4°C, Hämatokritwert = 0,52. K = Blut einer normalen Ratte, 6 Tage bei 4°C, Hämatokritwert = 0,45.

Kaliumeintrittes in der ersten Stunde beträgt bei den Kontrolltieren in meq K/l Zellen $6,63 \pm 1,81^6$ ($n = 13$), bei adrenaletomierten Tieren $11,36 \pm 3,35^6$ ($n = 11$). Die Unterschiede sind der grossen Streuung wegen nicht

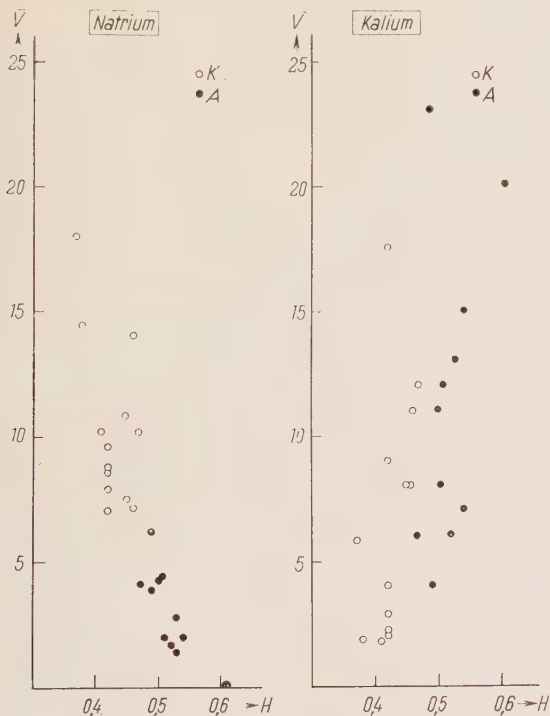


Abb. 2. Beziehung zwischen aktivem Natrium- bzw. Kaliumtransport und Hämatokritwert. Ordinate: $\bar{V}$ = mittlere Geschwindigkeit des Natriumaustrittes (links) bzw. des Kaliumeintrittes (rechts) in der ersten Stunde in meq/l Zellen. Abszisse: H = Hämatokritwert. A = adrenaletomierte Tiere. K = Kontrolltiere.

signifikant ($P > 0,05$). Auch in Versuchen mit Blut adrenaletomierter Tiere, bei welchen praktisch kein aktiver Natriumtransport mehr zu beobachten war,

7 H. A. KUNZ und F. SULSER, *Helv. physiol. Acta* 1958, im Druck.
8 D. M. WOODBURY und A. KOCH, *Proc. Soc. exp. Biol. Med.* 94, 720 (1957).
9 A. GROLLMAN, *Amer. J. Physiol.* 179, 36 (1954).
10 H. J. SCHATZMANN, *Exper.* 10, 189 (1954).
11 E. SHERWOOD JONES, *Nature* 176, 269 (1955); *Exper.* 14, 72 (1958).
12 F. SULSER und R. GANTENBEIN, in Vorbereitung. – J. BRONSTEIN, F. SULSER, H. A. KUNZ und W. WILBRANDT, *Helv. physiol. Acta* 1958, im Druck.

Antigenic Studies on Human Plasminogen

Although the human serum protease, plasmin (fibrinolysin), and its inactive precursor, plasminogen (profibrinolysin), have been studied extensively¹, attempts to determine the antigenic properties of plasminogen preparations have not been reported. This communication presents evidence for the inhibition of the proteolytic activity of streptokinase (SK)-activated human plasminogen by rabbit antiserum prepared against purified human plasminogen (PHP) and for the antigenic heterogeneity of the plasminogen preparation.

Human plasminogen was prepared according to KLINE² from Human Fraction III and lyophilized. The final preparation contained 90 μ g N/mg, and 25 μ g N of this material, when activated by SK, had a proteolytic activity equivalent to that of 11 μ g crystalline trypsin (Worthington). The major part of this preparation migrated as a single component on paper electrophoresis at pH 8.6 in 0.075 μ Veronal buffer; however, 10–15% appeared as a second component constantly.

Two adult New Zealand white rabbits were injected intravenously with 5 mg PHP at weekly intervals for three weeks. Samples of serum at the fourth week indicated precipitin titers of only 1:100 by the ring test. At this time 1 ml of a preparation of PHP in the FREUND and McDERMOTT³ adjuvant (8.4 mg PHP/ml) was injected subcutaneously. After two weekly injections of this antigen the serum from each animal gave a precipitin titer of 1:10 000. These animals, as well as two similar untreated animals, were exsanguinated; the pooled sera were designated, respectively, immune rabbit serum (IRS) and normal rabbit serum (NRS).

Each of these pools of serum was tested for its effect on the proteolytic activity of SK-activated PHP by the casein digestion method previously described⁴. Essentially, this method consists in the determination of trichloroacetic acid soluble products in the digest by the FOLIN-CIOALTEAU phenol procedure. Color values were read at 650 $m\mu$ in a Lumetron Colorimeter. Table I shows an increased proteolytic activity over that exhibited by PHP alone in the PHP-serum mixtures containing either IRS or NRS. Less activity was recorded with the PHP-IRS mixture than with the PHP-NRS, but the results did not conclusively demonstrate an inhibition by the rabbit antiserum. These findings were to be expected, since whole rabbit serum itself contains considerable plasminogen which is activated by PHP-SK mixtures⁵.

To eliminate the complicating effects of the presence in whole serum of rabbit plasminogen, both the IRS and NRS were fractionated by the ethanol method of NICHOL and DEUTSCH⁶. The rabbit proteolytic proenzyme was found to be localized largely in their precipitate B (beta- and gamma₁-globulin) while the antibody-containing fraction, precipitate C (gamma₂-globulin) was virtually free of rabbit plasminogen. (Subsequent studies in this laboratory by continuous flow electrophoresis of rabbit serum have confirmed the occurrence of plasminogen in the beta-

gamma₁-globulin area. It is significant that no normal inhibitors for rabbit or human plasmin were observed in the slower migrating gamma-globulins.)

Table I

Effect of whole normal and immune rabbit sera and of fractions therefrom on the proteolytic activity of SK-activated human plasminogen

Contents of Digestion Mixture ⁷	Optical Density
PHP ⁸	0.370
PHP + immune rabbit serum (IRS) (0.4 ml)	0.505
PHP + normal rabbit serum (NRS) (0.4 ml)	0.710
PHP + ppt B ⁹ from NRS (0.684 mg N)	too high to read
PHP + ppt B from IRS (0.696 mg N)	too high to read
PHP + ppt C ⁹ from NRS (0.752 mg N)	0.370
PHP + ppt C from IRS (0.776 mg N)	0.130
PHP + ppt C from IRS (0.752 mg N) (incubated 30 min at 37°C before activation with SK)	0.115

A repetition of tests with the casein digestion procedure, using the immune gamma-globulin (IGG) and normal gamma-globulin (NGG), in place of the original whole sera, showed that the IGG exhibited a definite inhibitory action on the proteolytic activity of SK-activated PHP, while the NGG did not affect the result (Table I). Specific precipitates were formed with IGG and not with NGG. The precipitates were not centrifuged off before enzyme assays, and hence it was demonstrated that the plasminogen in the precipitated complex either could not be activated by SK, or the activity of the plasmin formed was inhibited. Incubation of IGG and PHP for 30 min at 37°C before activation reduced the final activity only about 3%. Similar results were obtained at other antibody concentrations suggesting that the major activity of the antibody was to inhibit plasmin rather than its activation by SK. However, further study of this point is required to elucidate this mechanism.

It was necessary to prepare a new lot of antiserum to proceed with further quantitative studies. The materials used were as described above: 5 rabbits were immunized by three weekly subcutaneous injections of PHP in the FREUND-McDERMOTT adjuvant (total, 25 mg PHP/animal). Pooled serum from these animals, collected on the fourth week, had a 1:5000 precipitin titer by the ring test. A pooled serum sample collected from 5 normal animals was demonstrated to have no precipitins for PHP. Both pools of sera were fractionated as above to obtain normal gamma-globulin (NGG) and immune gamma-globulin (IGG) as the NICHOL-DEUTSCH precipitate C. (This fractionation procedure has been subsequently used extensively in this laboratory to obtain rabbit plasminogen preparations. In several instances precipitate C has contained some proteolytic activity which could be activated by SK-human plasminogen preparations.)

¹ S. MULLERTZ, *Acta physiol. scand.* **38**, suppl. 130 (1956). - S. SHERRY and N. ALKJAERISG, *Ann. N. Y. Acad. Sci.* **68**, 52 (1957).

² D. L. KLINE, *J. biol. Chem.* **204**, 949 (1953).

³ J. FREUND and K. McDERMOTT, *Proc. Soc. exp. Biol. Med. N. Y.* **49**, 548 (1942).

⁴ W. M. MEYERS and K. L. BURDON, *Arch. Biochem. Biophys.* **62**, 6 (1956).

⁵ W. M. MEYERS and K. L. BURDON, *Amer. J. Physiol.* **190**, 303 (1957).

⁶ J. C. NICHOL and H. F. DEUTSCH, *J. Amer. chem. Soc.* **70**, 80 (1948).

⁷ One ml 6% casein (Hammarsten quality) and 0.2 ml Varidase (500 units SK), in addition to the indicated components, were included in the digestion mixture. All reagents were prepared in *M*/10 phosphate buffer (pH 7.8) and the total volume was 3 ml. Incubation was at 37°C for 30 min. Except where stated the serum or its fractions and SK were added simultaneously.

⁸ Purified Human Plasminogen from Human Fraction III by method of KLINE². All digests contained 33 μ g PHP Nitrogen.

⁹ Prepared by ethanol fractionation method of NICHOL and DEUTSCH⁶.

Results of further tests with these materials are summarized in Table II. Since the PHP was only approximately 50% soluble under the conditions used, it was necessary to conduct quantitative assays with the soluble portion only. However, the proteolytic activity of the soluble and insoluble portions of this preparation per mg nitrogen were essentially the same. The indicated amounts of soluble PHP were mixed, in duplicate, with 1 ml of 2% NGG or 1 ml of 2% IGG in a total volume of 3 ml. All solutions were prepared in *M*/10 phosphate buffer at pH 7·8, centrifuged at high speed, and filtered to remove insoluble material. The PHP-globulin preparations were incubated 30 min at 37°C and then kept at 4°C for 24 h. At the end of this time all tubes were centrifuged at 2–4°C and the supernatants saved for enzymatic analysis and checks for antigen (An) or antibody (Ab) excess by the ring test. The specific precipitates were washed three times with cold 0·15 *M* NaCl and total nitrogens determined (micro-Kjeldahl). (No precipitate was obtained with the NGG and hence no values on this are included in Table II.) Proteolytic activity assays were carried out on the supernatants and on a solution containing corresponding amounts of soluble PHP only.

Table II
Effect of normal and specific immune rabbit gamma-globulins on Human plasminogen*

μg Plas- mino- gen N	Proteolytic Activity (Opt. Density)		Antigen (An)- Antibody (Ab) analyses		
	Plasmino- gen only	Supernatant Plasmino- gen + NGG	Plasmino- gen + IGG	μg N in An-Ab ppt.	Superna- tant Com- position
5·3	0·040	0·040	0·002	139	Ab
10·6	0·080	0·085	0·035	241	Ab, An
15·9	0·200	0·210	0·050	296	Ab, An
26·5	0·330	0·335	0·110	352	Ab, An
42·4	0·560	0·570	0·270	360	An

* Conditions of assay given in text.

The data (Table II) indicate that the presence of IGG reduced the proteolytic activity of the supernatants markedly, while NGG had no effect. The portion of the plasminogen preparation responsible for proteolytic activity could be virtually completely precipitated in the region of large Ab excess. The results further suggested that, since both An and Ab were present in the supernatants over a broad zone, PHP probably contains more than one antigenic component. This was confirmed by using the OUDIN agar-diffusion technique¹⁰ which demonstrated that the soluble PHP contained at least 4 antigenic components reacting with IGG. When three whole normal human sera were checked individually against IGG, multiple rings were also formed.

It was of interest to determine if IGG affected human blood clot lysis. One ml of 2% IGG in saline was mixed with 1 ml whole fresh human blood and allowed to clot. Streptokinase (1000 units) was injected into the center of these clots, as well as into two sets of control clots, one containing only saline and blood and the other 2% NGG and blood. Clot lysis was virtually complete after 90 min in the control tubes, while in those containing IGG very little lysis had taken place after 24 h. Although this is an extremely crude procedure it did furnish evidence for the inhibition of fibrinolysis in whole blood by IGG.

Thus the KLINE procedures for preparation of PHP yielded immunologically heterogeneous preparations; however, a specific antiserum was obtained which inhibited the proteolytic and fibrinolytic activity of human plasmin and formed specific precipitates. This heterogeneity makes further immunochemical studies on this plasminogen preparation of doubtful significance and renders an immunochemical assay for plasminogen impossible at this time.

We wish to thank Hyland Laboratories, Los Angeles, and Lederle Laboratories, Division American Cyanamid Company, Pearl River, N. Y., for the generous supply of Human Fraction III and Varidase, respectively. This study was supported by a grant from the National Institutes of Health (E-1563), Public Health Service, and a Summer Fellowship (W.M.M.) from the American Foundation for Allergic Diseases.

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Résumé

Les auteurs ont préparé, chez le lapin, les anticorps spécifiques inhibiteurs des activités fibrinolytiques et protéolytiques du plasminogène humain activé par la streptokinase. Le plasminogène humain purifié est un antigène hétérogène.

Cholesterol Blood Levels in Rh Sensitized
Women Treated with Rh Hapten

In this laboratory we are engaged in an attempt to assess the value of oral administration to the Rh negative sensitized woman of the Rh hapten (a red cell fraction described previously by CARTER¹ and CARTER *et al.*²). Although several workers, including HOWE and RUSTIGIAN³ and OSBORN⁴, have reported inability to confirm *in vitro* observations on the Rh hapten, this inability due, it would seem, to technical variations, BARNARD⁵ and GOLDSMITH⁶ independently have successfully confirmed this phase of the work. WOLF *et al.*⁷ stated: 'A number of our observations suggest that the term Rh hapten may correctly be applied to the extract described by CARTER'. Although WOLF *et al.*⁷, MARSTERS *et al.*⁸, and SPURLING *et al.*⁹ have been unsuccessful in the clinical use of Rh hapten to prevent erythroblastosis, these failures due to use of too little of the fraction too late in pregnancy, EHRENBERG¹⁰ and SCHUBERT and GRUNBERG¹¹ have reported clinical successes.

¹ B. B. CARTER, *Amer. J. clin. Path.* 17, 646 (1947); *J. Immunol.* 61, 79 (1949); *Amer. J. clin. Path.* 21, 561 (1951); *Lancet* 1954, 1267.

² B. B. CARTER, A. C. WILLIAMSON, J. LOUGHREY, and C. H. INGRAM, Jr., *Amer. J. Obstet. Gynec.* 72, 655 (1956).

³ C. HOWE and R. RUSTIGIAN, *J. Immunol.* 64, 505 (1950).

⁴ D. A. OSBORN, *J. clin. Path.* 4, 470 (1951).

⁵ R. D. BARNARD, *Proceedings of the Vth International Congress on Blood Transfusion*, p. 88 (1955).

⁶ J. W. GOLDSMITH, *Amer. J. Obstet. Gynec.* 59, 172 (1950).

⁷ A. M. WOLF, C. SCHLUTZ, M. FREUNDLICH, and S. O. LEVINSON, *J. Amer. med. Ass.* 144, 88 (1950).

⁸ R. W. MARSTERS, R. T. F. SCHMIDT, and M. E. BLACK, *Amer. J. Obstet. Gynec.* 63, 549 (1952).

⁹ C. L. SPURLING, M. S. SACKS, and E. F. JAHN, *Blood* 5, 1125 (1950).

¹⁰ C. J. EHRENBERG, *J. Lancet* 75, 275 (1955).

¹¹ J. SCHUBERT and A. GRUNBERG, *Pediatricke listy* 5, 1 (1950).

¹⁰ J. OUDIN, *Meth. med. Res.* 5, 335 (1952).

Table I
Cholesterol Blood Levels, mg/100 ml Blood

Patient	T.P.*	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Test 7	Test 8	
A	×	466	335	423	232	249	209	348	214	
B	×	288	213	262	177	199	265	209		
C	×	217	224	191	299	244	226	315		
D	×	199	171	199	174	166	171	179		
E	×	137	216	156	173					
F	×	216	194	181						
G	×	166								
H	.	178								

* T.P. refers to treated, pregnant women.

Table II
Cholesterol Blood Levels, mg/100 ml Blood

Patient	T.N.P.*	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Test 7	Test 8
A	×	288	214	184	415	194	178	169	249
B	×	294	166	216	173	209			
C	×	183	204	177	257	222			
D	×	156	166	163	166	179			

* T.N.P. refers to treated, non-pregnant women.

The Rh hapten, produced as described elsewhere⁹ and made convenient to handle by mixing with powdered lactose, is taken up into gelatin capsules in aliquots of not less than 100 mg. It is being supplied at present only to Rh negative, sensitized women who have lost one or more demonstrably erythroblastotic babies and who have homozygous husbands, but who are not yet pregnant again. The physician provides treatment until the Rh antibody titer is at zero when he recommends pregnancy. The hapten is supplied throughout pregnancy. Following this regimen, with patients possessing the criteria described above, in a steadily enlarging series (which will be reported elsewhere) there has been not one infant lost over a three year period. A paper describing the antibody response to oral administration is in press in the American Journal of Obstetrics and Gynecology.

In the process of investigating the effects of oral administration, we have been curious about the effect of the hapten on blood cholesterol levels. The crude fraction in use at present contains more than 35% cholesterol and/or other sterols. Modern medical interest has centered on the relation of cholesterol to atherosclerosis. Since dosage of the Rh hapten by mouth has not exceeded 200 mg daily, over eight to twelve months' periods, it was not supposed that this small quantity of exogenous cholesterol *per se* would in any way affect blood levels. However, we considered it at least possible that the administration of the Rh hapten might stimulate the production of endogenous cholesterol.

CHERNICK *et al.*¹² have demonstrated that cholesterol can be synthesized in the arterial wall. Hypercholesterolemia appears in many cases to be concomitant with atherosclerosis. The causal relationship is not clear. Nevertheless, MORETON¹³ has shown that coarsely suspended substances of many kinds, as well as large fatty particles in lipemia, are removed from the blood and retained in the intima by the reticuloendothelial cells. Hypothetically, according to DEUEL¹⁴, the protein in the ar-

terial lining in atherosclerosis is altered from cholestero-phobic to cholesterophilic substances. Presumably, union of Rh antibody with hapten could provide suspended material in the blood which might be retained in the intima and favor cholesterol deposition and/or cholesterol synthesis.

We made tests for serum cholesterol levels on each blood received from sensitized Rh negative women being given Rh hapten by mouth for a total of 61 individual bloods. This group of bloods included 38 specimens from pregnant, treated women and 23 from non-pregnant treated women. The study extended over a six months' period, during which time these women received from one to two 100 mg capsules Rh hapten daily. Bleedings were taken from these women biweekly for study of both Rh antibody titers and serum cholesterol levels.

Table III
Cholesterol Blood Levels, mg/100 ml Blood

Donor	P.N.T.	Test
A	×	274
B	×	332
C	×	194
D	×	171
E	×	873
F	×	236
P.N.T. refers to pregnant, non-treated women	G N.P.	262
H	×	294
N.P. refers to non-pregnant women	I	184
J	×	171
K	Normal	144
Normal refers to normal males	L	156
M	×	163
N	×	179
O	×	154
P	×	185
Q	×	136

¹² S. CHERNICK, P. A. SRERE, and I. L. CHAIKOFF, *J. biol. Chem.* 179, 113 (1949).

¹³ J. R. MORETON, *Science* 107, 371 (1948).

¹⁴ H. J. DEUEL, *The Lipids*, vol. 2 (Interscience, New York 1955).

For controls we made tests for serum cholesterol levels on 17 bloods from individuals neither receiving nor having received Rh hapten in any form. This group included four bloods from non-pregnant women, six bloods from preg-

nant, untreated women and seven bloods from normal males. This seemed a sufficient number of control subjects since the normal range in cholesterol levels is firmly established and we sought only to check our own methods.

The method used in testing for serum cholesterol blood levels was that described by SCHOENHEIMER and SPERRY¹⁵. The proteins are precipitated from the serum by an acetone-alcohol mixture and simultaneously the cholesterol and cholesterol esters are extracted. Digitonin is used to precipitate the cholesterol after saponification and this is tested as to color development with acetic anhydride-sulfuric acid reagent, in comparison with color produced in a standard cholesterol solution. Color readings were made with a Bausch and Lomb spectrophotometer. HAWK, OSER, and SUMMERSON¹⁶ give a range of 150 to 300 mg cholesterol in 100 ml blood as normal.

As will be seen from Table I, only five tests made on bloods from treated, pregnant women exceeded the normal range. This is of interest since DEUEL¹⁴ states that cholesterol levels are normally higher in pregnancy. Four of these tests are on bloods from the same woman, drawn at different times. In Table II, which deals with treated non-pregnant women, there is one specimen of blood in which the normal cholesterol range is exceeded. Among the pregnant, non-treated group (Table III) there are two bloods which exceed normal levels. One of these findings, at a level of 873 mg cholesterol, was confirmed in another laboratory. None of the normal bloods exceed the range for cholesterol.

Apparently the oral administration of Rh hapten does not affect serum cholesterol levels. Instead, there was a certain amount of fluctuation from one test to another on the same individual, nearly all of these within the normal range.

In summary, 61 bloods from Rh sensitized women treated with Rh hapten given orally were tested for serum cholesterol levels, using 17 control bloods from pregnant, non-treated women, non-pregnant women and normal males. Differences in cholesterol levels between the treated and control groups were not significant.

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Zusammenfassung

Cholesterin lässt sich als Bestandteil des ungereinigten Rh-Haptens nachweisen. Deshalb wurde geprüft, ob die perorale Aufnahme dieser Fraktion den Blutcholesterinspiegel beeinflusst. Bei 17 Menschen, die kein Hapten erhielten, und bei 61 Hapten-Empfangenden wurde keine Erhöhung des Cholesterinspiegels festgestellt.

¹⁵ R. SCHOENHEIMER and W. M. SPERRY, *J. biol. Chem.* **106**, 745 (1934).

¹⁶ P. B. HAWK, B. L. OSER, and W. H. SUMMERSON, *Practical Physiological Chemistry* (Blakiston, New York 1954).

energy for the red cells¹. Mature erythrocytes utilize glucose mainly by means of glycolysis, i.e. by anaerobic fermentation to pyruvic and lactic acids, only a very small fraction being oxydized aerobically. The hexose-monophosphate shunt has been recently demonstrated to occur also in the red cells and appears to be an important metabolic pathway². Abnormalities of the glycolysis and related enzymes have been described in the *in vitro* storage as well as in a number of hemolytic conditions and are likely to occur also during the *in vivo* ageing.

But the ultimate significance of glycolysis deserves further study. Among the many problems which have still to be answered, an important point is the following: do red cells utilize a fixed amount of glucose for their metabolic requirements regardless of external factors? Or do they have to be considered as simple enzyme parcels passively reacting in a peripheral medium and metabolizing any suitable substrate? Or might their metabolic activity be influenced by the environmental conditions and adjust itself to plasma composition?

The statement generally reported, that the glycolysis rate does not depend upon the initial glucose level within normal ranges, would support the first possibility aforementioned. In the present paper some contradictory results are reported.

Materials and methods.—The investigations have been carried out in normal individuals ranging from 22 to 35 years of age. Glycolysis has been determined according to HOLLINGSWORTH's technique³. Blood sugar was measured in duplicate before incubation, and after 1 and 2 h, by means of the SOMOGY-NELSON's method. The standard error for the determination of glucose in duplicate was ± 2.7 mg%.

Results.—The samples were withdrawn 1–4 h after lunch. In the reconstituted samples, the red cell count ranged from 3 360 000 to 5 200 000 and the hemoglobin from 9.9 to 14.9 g%. The white cells were found between 0 and 800 per mm³. The RBC: WC ratio ranged from 4000 to 50 000. No changes of the hematocrit were observed at the end of the experiments.

The mean initial glucose level was 76.2 mg% with a standard deviation of ± 17.9 . It dropped to 40.2 mg% ± 11.4 after 2 h. Glycolysis, expressed as mg of glucose utilized by 100 ml of packed red cells per hour, has been found to be 43.9 mg with a standard deviation of ± 13.6 . The percentage in respect to the initial glucose level was 23.7 ± 4.6 .

By plotting, on a normal graphic, the glycolysis values against the initial glucose level, a fairly good correlation was observed and confirmed statistically. In our 40 normal controls, the correlation coefficient (r) was found to be $+0.742$ and the calculated ' t ' value (6.822) was highly significant ($P < 0.001$; Figure). The regression line is given by the formula: $y = 7.4 + 0.48x$, where y is the expected glycolysis and x the initial glucose level. The confidence limits (2σ) are ± 15 .

According to these data, glycolysis could be tentatively interpreted as a first order enzymatic reaction. Since, in this case, the amount of substrate (S) present at the time t is given by the formula: $S_t = S_0 e^{-kt}$, k being the velocity constant, the reaction itself could be better expressed

Influence of Blood Sugar Level on the Glycolytic Activity of Human Red Cells

Considerable evidence has been collected in the last few years on the importance of glucose as the main source of

¹ O. F. DENSTEDT in J. L. TULLIS, *Blood cells and plasma proteins* (Academic Press Inc., New York 1953), p. 223. – S. GRANICK, *Blood* **4**, 404 (1949). – T. A. J. PRANKERD, *Brit. J. Haematol.* **1**, 131 (1955).

² O. F. DENSTEDT, in J. L. TULLIS, *Blood cells and plasma proteins* (Academic Press Inc., New York 1953), p. 223.

³ J. W. HOLLINGSWORTH, *J. Lab. clin. Med.* **45**, 920 (1955).

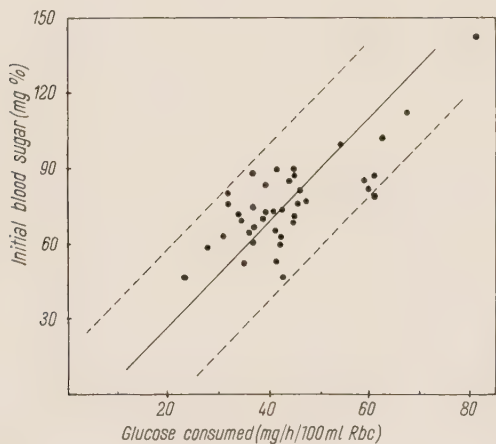
Modifications of glycolysis during glucose tolerance test

Case	Time 0		After 30 min		After 60 min		After 120 min	
	Initial glucose	Glycolysis	Initial glucose	Glycolysis	Initial glucose	Glycolysis	Initial glucose	Glycolysis
1	75	55	—	—	47	44	51	46
2	137	76	142	41	95	45	84	32
3	108	61	116	66	—	—	75	39
4	78	42	115	51	98	32	70	84
5	79	39	120	38	137	36	93	28
6	90	61	84	63	118	45	59	42
7	74	43	176	47	132	30	—	—

Glycolysis is expressed as mg of glucose/100 ml RBC/h.

by the formula: $k = \frac{\log N_0 - \log N_t}{t} \times 2.303$.

Since a perfect linear correlation has been found between the k and the hematocrit of samples with different RBC: plasma ratio, in order to have comparable results, the values of k have been corrected for an hematocrit of 50. In the 40 normal subjects of our group, the velocity constant/h was 0.0066 ± 0.0018 . A relatively slower speed of the reaction was found in the first hour presumably due to some delay in the equilibration of the temperature between blood and waterbath.



Correlation between initial glucose level and glycolysis. Solid line is the calculated regression line for the points shown and the dashed lines the 2 σ confidence limits.

In order to confirm the existence of a correlation between the initial glucose level and the rate of glycolysis, experiments were carried out both *in vivo* and *in vitro*. By adding *in vitro* measured amounts of glucose to samples of blood, no changes of the glycolysis were observed as compared with that of the blood alone. Glycolysis was also determined in normal individuals during a glucose tolerance test. 40–60 g of glucose were given by mouth and glycolysis was measured in the fasting state and after 30, 60, and 120 min. No consistent pattern was obtained. In three subjects (cases 1, 2, 3 of the Table) a fairly good correlation was found between the initial glucose level and the glycolysis: in the single cases the points approximated a straight line. In other three instances (cases 4, 5, 6 of the Table), the glycolysis rate changed quite unpredictably with no constant relationship to the variations of the blood sugar. No constant changes were observed in one case given 25 g of glucose intravenously (case 7 of the Table). It may be of interest to note that the subjects in

whom a correlation could be elicited were allowed to perform normal muscular activity during the test while the other four cases were kept resting in bed.

Discussion.—Our results expressed as mg/100 ml RBC/h are in perfect agreement with the ones reported by others with the same technique⁴.

As to the concentration of glucose, it is generally reported even in recent papers⁵ that the initial glucose level, if below 500 mg%, has no effect on the glycolysis whereas higher values inhibit it. But from earlier authors, it was reported that glycolysis could be considered as a first order reaction, where the amount of glucose utilized is proportional within the limits of the experimental error to the amount of the available substrate⁶. While the data concerning experiments *in vitro* are rather conflicting, the results *in vivo* appear more uniform, even if not all authors called attention to it⁷. The possible correlation between glycolysis and initial glucose level should be considered when glycolysis is followed under the influence of certain drugs or in different experimental conditions; in many instances the observed values would become not significant if expressed as velocity constant.

One wonders if the intimate mechanism of the changes of blood sugar concentration may account for the different behaviour of glycolysis. As a matter of fact, when glucose level was artificially increased by administration of sugar in resting patients, no constant modifications could be detected. In the cases in which the changes occurred during muscular activity, as also reported in animals in which the blood sugar was altered by endogenous mobilization, a fairly good correlation could be elicited.

On the basis of the available data, the concentration of glucose appears to be an important factor but not the only one. Further studies are in progress in order to ascertain the possible influence both *in vivo* and *in vitro* of some hormonal substances.

⁴ J. W. HOLLINGSWORTH, J. Lab. clin. Med. 45, 920 (1955). – J. G. SELWYN and J. V. DACIE, Blood 9, 414 (1954). – J. E. ULTMAN, G. A. HYMAN, J. L. HARVEY, and A. R. DENTE, Blood 12, 1114 (1957).
⁵ R. M. BIRD, J. biol. Chem. 169, 492 (1947). – J. W. HOLLINGSWORTH, J. Lab. clin. Med. 45, 920 (1955). – E. PONDER, Hemolysis and related phenomena (Grune & Stratton, New York 1948). – J. G. SELWYN and J. V. DACIE, Blood 9, 414 (1954). – J. E. ULTMAN, G. A. HYMAN, J. L. HARVEY, and A. R. DENTE, Blood 12, 1114 (1957).
⁶ A. KANITZ, Biochem. Z. 57, 437 (1913). – P. RONA and A. DÖBLIN, Biochem. Z. 32, 489 (1911). – P. RONA and G. G. WILENKO, Biochem. Z. 62, 1 (1914).
⁷ C. COLIZZI and G. TUSINI, Atti Soc. lombarda Sci. med. Biol. 8, 238 (1953). – F. VACCARI and M. ROSSANDA, Arch. int. Pharmacodyn. 90, 421 (1952). – F. VACCARI, M. ROSSANDA, and G. MALAGUTI, Arch. int. Pharmacodyn. 99, 234 (1954).

By expressing the glycolysis through the velocity constant corrected for the hematocrit, the variations due to the concentration of glucose are automatically eliminated. However, it has to be kept in mind that glycolysis is not a simple process but consists of a chain of interrelated reactions and therefore the use of a single velocity constant may be an oversimplification which is not free from reasonable criticism.

Whereas the relationship between initial glucose level and glycolysis is strongly suggestive of a first order reaction, the disappearance of the glucose followed at shorter intervals during the first 2 h approximates to a straight line more consistent with a reaction of order zero. We have no satisfactory explanation for this discrepancy.

Since under normal circumstances the range of the sugar levels in the blood is relatively small, no significant error can be made if one expresses the glycolysis in the conventional way. However, this method becomes unreliable when the glycolysis is followed during spontaneous or induced sugar variations.

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Riassunto

Studiando la glicolisi di globuli rossi umani è stata notata una correlazione statisticamente significativa tra livello iniziale di glucosio e glicolisi, espressa in mg di glucosio utilizzati da 100 ml di globuli rossi per ora. In condizioni di carico sperimentale con glucosio la correlazione ha avuto un comportamento variabile.

Activation of Cathepsin in Fatty Liver

It has been shown in recent years that cathepsin activity of liver cells is mainly located within mitochondria¹. DE DUVE *et al.*² have found that the cathepsin which is active on haemoglobin is bound to a special type of particles, which have the same sedimentation characteristics such as light mitochondria. These particles have been called *lysosomes*. They contain practically all acid phosphatase, β -glucuronidase, ribonuclease, desoxyribonuclease and cathepsin activities of the liver cells. The activity of these enzymes is very low when the lysosomes are intact, but increase many times as a result of damaging treatments. These also produce the displacement of the bound enzymes from the particles to the suspension fluid. Many different treatments produce similar effects: homogenization in Waring blender, suspension in hypotonic solutions, incubation at 37°C for a short time, addition of salts, addition of substances which decrease the surface tension.

The presence of mitochondrial damage in fatty livers has been described³. Morphological modifications of mito-

chondria consist mainly in the swelling of the particles. Swollen mitochondria show higher permeabilities to many substances originally contained inside their body, as ribonucleic acid⁴, cytochrome *c*⁵, pyridine nucleotides⁶, adenosine phosphates⁷, thiamine pyrophosphate⁸, citrate⁹. These substances are displaced from the mitochondrial body to the suspension fluid as a consequence of treatments which produce mitochondrial swelling. These treatments resemble strongly those which produce lysosome damage. It seems then not improbable that lysosome damage exists in fatty livers. Strong increase of acid phosphatase activity of fatty livers has been reported¹⁰. In these conditions, a redistribution of the enzyme takes place, as a large part of it is displaced from mitochondria into the fluid part of the homogenate. An increase of β -glucuronidase in fatty livers has been reported by MILLS *et al.*¹¹.

Thus it was interesting to study the behaviour of cathepsin, another enzyme present in lysosomes. Albino rats weighing 150–180 g were used. They were fed on a standard diet. Fatty infiltration of the liver was obtained either by 2 subcutaneous injections of carbon tetrachloride (0.2 ml of a 20% solution in olive oil, each day), or also by one subcutaneous injection of white phosphorus (0.1 ml of the 0.5% solution in olive oil). The rats were killed by decapitation 24 h after the last injection. The liver was immediately dissected and transferred to a cold room at 2°C.

10% homogenates were prepared in a Potter-Elvehjem homogenizer, two types of tissue suspensions being prepared in each case: the first one was prepared with 0.25 *M* sucrose, the second one with 0.25 *M* sucrose containing 0.1% *Triton X-100* (obtained through the courtesy of Prof. H. G. K. WESTENBRINK). This was used to produce maximum activation of cathepsin, as a result of the disintegration of the particles (WATTIAUX and DE DUVE¹²). In order to avoid mechanical damage to the particles in the case of the homogenates prepared with 0.25 *M* sucrose, mitochondria were not isolated and the enzyme determinations were performed on the whole homogenates. These were used within 10 min after the death of the animal.

Cathepsin activity was determined at 37°C by a modification of the method of ANSON¹³ described by GIANETTO and DE DUVE¹⁴. The reaction mixture had the following composition: 0.17 *M* acetate buffer, pH 5.0, 0.00026 *M* haemoglobin, the enzyme and 0.25 *M* sucrose to 3 ml. The reaction was stopped by adding 4 ml of ice-cold 0.3 *M* trichloroacetic acid. The mixture was cooled immediately and filtered in the ice box, in order to decrease the hydrolysis of sucrose, the products of which interfere with the

¹ M. U. DIANZANI, *Biochim. biophys. Acta* 11, 353 (1953).

² M. U. DIANZANI and I. VITI, *Biochem. J.* 59, 141 (1955).

³ G. S. CHRISTIE and J. D. JUDAH, *Proc. Roy. Soc. (London)* [B] 907, 241 (1954). – M. U. DIANZANI, *Biochim. biophys. Acta* 17, 391 (1955). – F. E. HUNTER and L. FORD, *J. biol. Chem.* 216, 357 (1955). – P. SIEKEVITZ and V. R. POTTER, *J. biol. Chem.* 215, 221 (1955). – G. F. MALEY and H. A. LARDY, *J. biol. Chem.* 215, 377 (1955).

⁴ H. BLASCHKO, G. V. R. BORN, A. D'IORIO, and N. R. EADE, *Biochem. J.* 62, 18 P (1956). – M. U. DIANZANI, *Biochem. J.* 65, 116 (1957).

⁵ M. U. DIANZANI and M. A. DIANZANI MOR, *Biochim. biophys. Acta* 24, 564 (1957).

⁶ W. C. SCHNEIDER, M. STRIEBICH, and G. H. HOGEBOOM, *J. biol. Chem.* 222, 969 (1956).

⁷ M. U. DIANZANI, *Biochim. biophys. Acta* 14, 514 (1954).

⁸ G. T. MILLS, J. PAUL, and E. E. B. SMITH, *Biochem. J.* 53, 245 (1953).

⁹ R. WATTIAUX and C. DE DUVE, *Biochem. J.* 63, 606 (1956).

¹⁰ M. L. ANSON, *J. gen. Physiol.* 20, 565 (1937).

¹¹ R. GIANETTO and C. DE DUVE, *Biochem. J.* 59, 433 (1955).

¹ M. E. MAVER and A. E. GRECO, *J. nat. Cancer Inst.* 12, 37 (1951). – J. T. FINKENSTADT, *Proc. Soc. exp. Biol. Med. N. Y.* 95, 302 (1957).

² C. DE DUVE, R. GIANETTO, F. APPELMANS, and R. WATTIAUX, *Nature* 172, 1143 (1953). – R. GIANETTO and C. DE DUVE, *Biochem. J.* 59, 433 (1955).

³ H. C. ZOLLINGER, *Revue Hématol.* 5, 696 (1950). – M. U. DIANZANI, *G. Biochim.* 2, 180 (1953). – M. U. DIANZANI and G. F. BAHR, *Acta path. microbiol. scand.* 35, 25 (1954). – E. LEVIN, R. M. JOHNSON, and S. ALBERT, *J. biol. Chem.* 228, 15 (1957).

Cathepsin activity of the liver of normal rats and of rats treated with steatogenic poisons.
The standard deviation is indicated after each average. Fat content of normal liver was mg 40.8 ± 4.7/g wet tissue. Those of the liver of the rats treated with CCl₄ or with phosphorus were respectively mg 71.1 ± 8.6 and 63.0 ± 7.8.

Amount of enzyme used mg	Normal rats		Rats treated with CCl ₄		Rats treated with phosphorus	
	µg tyrosine set free in 8 min	µg tyrosine /mg N	µg tyrosine set free in 8 min	µg tyrosine /mg N	µg tyrosine set free in 8 min	µg tyrosine /mg N
Without Triton 20 .	0	0	7.0 ± 7.0	14.4 ± 14.4	4.6 ± 4.0	9.3 ± 9.0
With Triton 20 .	32.7 ± 19.6	57.5 ± 32.3	54.5 ± 19.8	121.7 ± 47.1	58.2 ± 22.6	118.8 ± 50.5
Stimulation % by Triton . .	∞	∞	678.5		116.5	
Without Triton 50 .	7.3 ± 7.0	5.1 ± 5.0	31.4 ± 13.9	28.3 ± 13.5	26.5 ± 8.8	21.6 ± 7.5
With Triton 50 .	62.4 ± 18.3	44.1 ± 16.4	97.7 ± 19.3	78.0 ± 21.2	90.3 ± 23.3	81.2 ± 17.9
Stimulation by Triton	754.8		211.1		240.7	
Without Triton 100 .	14.3 ± 9.6	5.1 ± 3.1	46.2 ± 17.2	20.6 ± 6.2	43.3 ± 20.1	18.1 ± 10.0
With Triton 100 .	99.7 ± 18.8	34.0 ± 7.8	137.1 ± 48.1	61.6 ± 24.7	122.0 ± 29.2	50.9 ± 16.4
Stimulation % by Triton . .	583.2		196.7		181.7	

colour reaction. All the solutions used, with the exception of trichloroacetic acid, were made either in 0.25 M sucrose or in 0.25 M sucrose containing 0.1% Triton X-100. Haemoglobin was purified according to GIANETTO and DE DUVE¹⁴. Three different amounts of enzyme were used for each determination: 20, 50, and 100 mg respectively. For each series 2 samples were prepared: in the first one, the reaction was stopped 2 min after the beginning of the incubation, in the second one it was stopped after 10 min. The aromatic degradation products of haemoglobin were measured on the deproteinized filtrates by means of the Folin-Ciocalteu reagent, with tyrosine as standard. The differences between the readings found at 10 and 2 min were retained as a measure of the enzyme activity. The values are given in the Table as micrograms tyrosine set free in 8 min.

In all homogenates, nitrogen was determined by the usual microkjeldahl technique. Total fat content of each liver was determined by ethereal extraction of the dry powder of the organ in a Soxhlet apparatus.

The Table shows that, in the case of normal liver, practically no enzymatic activity was found in the homogenates prepared without Triton, when the amount of enzyme used corresponded to 20 and to 50 mg of tissue. The activity was present, but very small, only with 100 mg tissue. The addition of Triton to the homogenates produced a strong activation of cathepsin activity. As the values per mg nitrogen were higher with 20 mg tissue than with higher amounts of tissue, it seems probable that in the last case saturation of the enzyme is attained. In the case of fatty livers, the enzyme activity was practically nil with 20 mg tissue, but was evident with 50 mg and was rather high with 100 mg. The addition of Triton produced further stimulation, but this was many times lower than that observed in the case of normal livers. The enzymatic activity in the presence of Triton was higher in the case of fatty liver than in that of normal liver. The difference is quite striking if the values are given per mg nitrogen.

One may conclude from these results that the activity of cathepsin is increased in fatty livers determined by carbon tetrachloride and by white phosphorus. One of the reasons for this increase is probably the damage of lysosomes. This cannot, however, be the only cause for the increase, as it was found also in the experiments made in the presence of Triton X-100.

The morphology of lysosomes has not yet been well established. NOVIKOFF, BEAUFAY, and DE DUVE¹⁵ have

succeeded in isolating from rat-liver a lysosome-rich fraction, which mainly consisted of small mitochondria and of a peculiar type of particle, the 'dense body'. 'Dense bodies' have been tentatively identified with lysosomes. The morphology of 'dense bodies' closely resembles that which was described by RHODIN¹⁶ and by ROUILLER and BERNHARD¹⁷ for 'microbodies'. These are present also in normal liver, but increase strongly in fatty livers produced by carbon tetrachloride or also by partial hepatectomy¹⁸. An increase of mitochondria with a very small diameter, probably to be identified with microbodies, was described in fatty liver by carbon tetrachloride also by DIANZANI and BAHR¹⁹. These facts may be interesting in order to understand the nature of the increase of cathepsin in fatty liver.

E. MARTINI and M. U. DIANZANI

Institute of General Pathology, University of Genoa (Italy), May 9, 1958.

Riassunto

L'attività cateptica del fegato grasso ottenuto mediante trattamento parenterale con CCl₄ o con fosforo nel ratto è aumentata rispetto al normale. Nel normale, gli omogenati hanno una attività cateptica molto bassa, che aumenta di molte volte se si aggiunge al mezzo il Triton X-100. Nel fegato grasso, invece, si osserva una discreta attività cateptica già in assenza di Triton; questo determina un aumento dell'attività, ma in misura percentualmente minore che nel normale. In presenza di Triton, l'attività cateptica dell'omogenato di fegato grasso è superiore a quella del fegato normale. L'aumento dell'attività enzimatica non è quindi interamente attribuibile alla lesione delle particelle che contengono l'enzima.

¹⁶ J. RHODIN, *Correlation of the ultrastructural organization and function in normal and experimentally changed convolute tubule cells of the mouse kidney* (Stockholm 1954).
¹⁷ C. ROUILLER and W. BERNHARD, *J. biophys. biochem. Cytol.* 2, Suppl. 355 (1956).
¹⁸ H. GANSLER and C. ROUILLER, *Schweiz. Z. allg. Path. Bakt.* 19, 217 (1956).
¹⁹ M. U. DIANZANI and G. F. BAHR, *Acta path. microbiol. scand.* 35, 25 (1954).

¹⁵ A. B. NOVIKOFF, H. BEAUFAY, and C. DE DUVE, *J. biophys. biochem. Cytol.* 2, Suppl. 179 (1956).

Effect of Azo Dyes on Rat Serum Para-Phenylene Diamine Oxidase Activity

Recently, MILLER, MILLER, and FINGER¹ noted that whereas 4'-methyl-4-dimethylaminoazobenzene (4'-MeDMAAB) has feeble carcinogenicity, 4'-ethyl-4-dimethylaminoazobenzene (4'-EtDMAAB) is an extremely potent liver carcinogen for rats. The authors state that 'no large difference in simple chemical reactivity of these dyes would be expected and the reason (i.e. for the great difference in carcinogenicity) seemingly must be sought in decisive metabolic and steric differences'.

A striking difference has now been found in the effect of these azo dyes on the para-phenylene diamine (PPD) oxidase activity of rat serum. Adult male albino rats were tail-bled (~ 1.5 ml) under ether anaesthesia and the PPD oxidase activity of the fresh serum was determined by the method of RAVIN². 24 h later, pairs of rats received intraperitoneal injections of arachis oil (0.6 ml/100 g body weight [b.w.]) and of solutions in arachis oil (0.6 ml/100 g b.w.) of the powerful liver carcinogen 3'-methyl-4-dimethylaminoazobenzene (3'-MeDMAAB; 16.5 mg/100 g b.w.), of 4'-MeDMAAB (16.5 mg/100 g b.w.) and of 4'-EtDMAAB (17.5 mg/100 g b.w.). At intervals thereafter, serum PPD oxidase levels were determined. The results are given in Table I.

After arachis oil injection, rat serum PPD oxidase activity increased during 2-4 days but, by 21 days, the levels had returned to normal. The same effects were obtained with the feeble carcinogen, 4'-MeDMAAB. However, the powerful hepatocarcinogens, 3'-Me- and 4'-EtDMAAB strongly suppressed serum PPD oxidase activity during the 4-day period following injection.

Injections of arachis oil solutions of the powerful 'local' carcinogens, 3,4-benzpyrene and 20-methylcholanthrene (MC) and of the non-carcinogenic pyrene or the application of whole body X-irradiation failed to suppress rat serum PPD oxidase activity, and it is of interest that N,N-dimethyl-*p*-(1-naphthylazo) aniline which is known to be a rapid sarcoma-inducing agent for female rats³ but which has a long induction time for hepatoma formation⁴

was also inactive in this respect. Indications have been obtained that injection of MC into male rats which received simultaneously 3'-MeDMAAB, speeds the return of serum PPD oxidase levels to normal, and we have noted that the PPD oxidase levels of female rats injected with 3'-MeDMAAB return more quickly to normal than is the case with male rats. MC is known to protect rats against hepatocarcinogenesis by 3'-MeDMAAB⁵ and it has been found that male rats are more susceptible than females to the azo dye⁶.

In view of the above-mentioned results and unpublished observations for other azo dyes, it seems that there is a close correlation between the hepatocarcinogenicity of an azo dye for rats and its ability, after intraperitoneal injection, to lower and maintain at a low level serum PPD oxidase activity. Our results point to a possible method for rapid screening of azo hepatocarcinogens and indicate that these substances may attack the liver precursors of caeruloplasmin, the copper-containing serum globulin which is probably identical with PPD oxidase. Confirmation that copper metabolism is profoundly influenced by hepatocarcinogenic azo dyes has been found in the observations (see Table II) that 3 days after injection of 3'-MeDMAAB, 4'-MeDMAAB and arachis oil only, serum copper levels as determined by the method of GUBLER *et al.*⁷ are strongly depressed in adult female rats which received 3'-MeDMAAB, but not at all in those injected with the feeble carcinogen 4'-MeDMAAB or with arachis oil only.

Addition of copper salts to the diet is known to protect rats against hepatoma formation by 3'-MeDMAAB⁸ and it was this fact as well as the following considerations which prompted the present investigation of the action of azo dyes *in vivo* on some rat serum enzymes which depend for activity on trace elements such as copper. MILLER *et al.*¹ noted that an unsubstituted 2-position is one of the requirements for carcinogenic activity of azo dyes and earlier NYE and LUCK⁹ had obtained evidence that 3'-

¹ J. A. MILLER, E. C. MILLER, and G. C. FINGER, *Cancer Res.* **17**, 387 (1957).

² H. A. RAVIN, *Lancet* **1956**, 726.

³ A. S. MULAY and R. W. O'GARA, *J. nat. Cancer Inst.* **18**, 843 (1957).

⁴ A. S. MULAY and H. I. FIRMINER, *J. nat. Cancer Inst.* **13**, 35 (1952).

⁵ H. L. RICHARDSON and L. CUNNINGHAM, *Cancer Res.* **11**, 274 (1951).

⁶ H. W. RUMSFIELD, W. L. MILLER, and C. A. BAUMANN, *Cancer Res.* **11**, 814 (1951).

⁷ C. J. GUBLER, M. E. LAHEY, H. ASHENBRUCKER, G. E. CARTWRIGHT, and M. M. WINTROBE, *J. biol. Chem.* **196**, 209 (1952).

⁸ E. PEDRERO and F. L. KOZELKA, *Amer. med. Ass. Arch. Path.* **52**, 155 (1951). – H. J. KING, J. D. SPAIN, and C. C. CLAYTON, *J. Nutr.* **63**, 301 (1957).

⁹ W. NYE and J. M. LUCK, *Abstr. Amer. chem. Soc., Meeting Sept. 1953*, 11C.

Table I
Para-phenylene diamine oxidase activity² (expressed as optical density at 530 mμ) of serums from pairs of rats

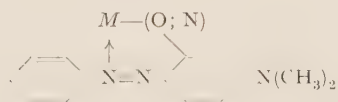
At time	Before and after injection of			
	Arachis oil only	Arachis oil solutions of		
		3'-MeDMAAB	4'-MeDMAAB	4'-EtDMAAB
24 h before injection	0.242; 0.369	0.369; 0.312	0.341; 0.328	0.327; 0.318
24 h after injection	0.332; 0.466	0.366; 0.297	0.418; 0.400	0.294; 0.285
48 h after injection	0.526; 0.598	0.263; 0.222	0.532; 0.548	0.254; 0.238
97 h after injection	0.394; 0.577	0.123; 0.171	0.532; 0.580	0.257; 0.187
21 days after injection	0.257; 0.341	died; 0.356	0.336; 0.348	0.336; 0.352

Serum (0.1 ml) is incubated with 2 ml of acetate buffer (pH 6) + 1 ml of PPD reagent (0.5 g PPD · 2 HCl/100 ml of water) for 1 h at 38° C. A serum-reagent blank containing in addition 1 ml of 0.1 % sodium azide was incubated at the same time. After reaction, 1 ml of 0.1% sodium azide was added to the first tube, the contents of both tubes were diluted to 10 ml with 3% sodium chloride and the colour read at 530 mμ (1-cm cell) in a Unicam SP 500 Spectrophotometer.

Table 11

Substance injected in arachis oil	Rat No.	Serum Copper Level ($\mu\text{g}/100\text{ ml}$)	
		24 h before injection	72 h after injection
3'-MeDMAAB	1	224	57
	2	281	30
Nil	3	174	191
	4	144	178
4'-MeDMAAB	5	154	241
	6	157	198

MeDMAAB becomes attached to proteins by linkage through its 2-position with a protein amino group. Since azoxy compounds are known to be transformed readily *in vitro* into *o*-hydroxyazo derivatives¹⁰, it was thought that similar reactions might occur *in vivo* leading to 2-hydroxy- and even 2-imino products of the metabolizing azo dyes. Molecules of this kind could then chelate with metals¹¹ (*M*):



a reaction which might be responsible for PPD oxidase inhibition.

As yet, no clear evidence has been obtained that such a mechanism is involved. Thus, 1-phenylazo-2-naphthol, a compound which could chelate with copper in the manner depicted, failed to inhibit rat serum PPD oxidase *in vivo*, and its solution in normal rat serum *in vitro* did not influence the oxidase activity of this serum. Crude metabolites of 3'-MeDMAAB from rat urine failed to inhibit rat serum PPD oxidase *in vitro* as did the parent azo dye, but in one experiment with 4'-EtDMAAB metabolites some inhibition was observed.

Injection of 8-hydroxyquinoline into rats stimulated strongly serum PPD oxidase activity. This compound like some *o*-aminophenolic substances, acts as a local carcinogen for the bladders of mice, and BOYLAND and WATSON¹² have suggested that a chelation mechanism may well be involved in this process. We have found that the hepatocarcinogen, 2-aminofluorene caused a feeble temporary decline in rat serum PPD oxidase activity, while its non-carcinogenic isomer, 4-aminofluorene was completely inactive. The potent liver carcinogen, 4-dimethylaminostilbene, proved to be as powerful an inhibitor of PPD oxidase *in vivo* as the azo carcinogen, 3'-MeDMAAB. Perhaps in the case of these amino carcinogens *o*-aminophenolic derivatives capable of chelation are produced in the liver.

Our results will be reported in detail elsewhere together with an account of the effects of azo carcinogens etc. on rat serum xanthine oxidase, an enzyme which requires molybdenum for its activity and which is known to be susceptible to inhibition by copper ions¹³.

¹⁰ G. M. BADGER and R. G. BUTTERY, J. chem. Soc. 1954, 2243.
¹¹ H. D. K. DREW and J. K. LANDQUIST, J. chem. Soc. 1938, 292. – M. L. ERNSBERGER and W. R. BRODE, J. org. Chem. 6, 331 (1941). – V. I. MUR, J. gen. Chem., Moscow (Consultants Bureau Translation) 26, 405 (1956).
¹² E. BOYLAND and G. WATSON, Nature 177, 837 (1956).
¹³ F. PASQUIER and J. POLONOVSKI, Bull. Soc. Chim. biol. 39, 1465 (1957).

The writer is indebted to the University of Sheffield for the James Morrison Fellowship in cancer research.

W. J. P. NEISH

Cancer Research Unit, The University, Sheffield, April 28, 1958.

Résumé

On a trouvé que les azoïques 3'-méthyl- et 4'-éthyl-4-diméthylaminoazobenzène, substances cancérigènes puissantes pour le foie du rat, empêchent fortement l'activité de la *p*-phénylènediamine oxydase du sérum sanguin. Au contraire, 4'-méthyl-4-diméthylaminoazobenzène avec pouvoir cancérigène très faible n'a aucune activité. L'azoïque 3'-méthyl-4-diméthylaminoazobenzène, mais pas son isomère 4'-méthyl, diminue fortement le cuivre sérique.

Über einen Cortison-äquivalenten Katalysator der Autoxydation der Linolsäure *in vitro*

Die nichtenzymatische Oxydationskatalyse der Linolsäure mit Cortison und anderen Corticosteroiden, als körpereigene Hormone, ist von praktischem und theoretischem Interesse¹. Das bei der Cortisonkatalyse der Linolsäure entstehende und mit der TBA-Reaktion proportional dem Sauerstoffverbrauch nachweisbare² Reaktionsprodukt ist, ebenso wie nach UV.-Bestrahlung dieser Fettsäure, ein Hydroperoxyd, dem biologische Bedeutung zukommt: Es ist bekannt, dass Linolsäurehydroperoxyd bestimmte Fermente, wie Succinoxidase, Cytochromoxydase und Cholinoydase zu hemmen vermag³, dass es die Atmung und Glykolyse von Ehrlichs Ascitestumorzellen hemmt⁴, dass es die Fähigkeit besitzt, Desoxyribonukleinsäure zu depolarisieren⁵, und dass es bestimmte Zellteilungen blockiert⁶. In diesem Zusammenhang ist erwähnenswert, dass die Oxydationskatalyse der Linolsäure mit Cortison, unter Bildung von Linolsäurehydroperoxyd, durch andere Steroide, solche mit Follikelhormonwirkung, gehemmt wird⁷.

Die Linolsäureoxydationskatalyse durch bestimmte Corticosteroide ist eine für deren Hormoncharakter ungewöhnliche Wirkung, deren Bedeutung für die Hormoneffekte unklar bleibt. Sie steht in Zusammenhang mit Metallkatalytischen Phänomenen, denn für ihr Zustandekommen sind Metallionen, besonders Cu²⁺, nötig, so dass ein Cortison-Cu-Komplex als massgeblich angenommen werden muss¹. Die Veresterung der Seitenkette der Corticosteroide hebt deren katalytische Wirkung auf. Diese ist somit für die Katalyse massgebend. Ein Beweis hierfür sind auch unsere Befunde, dass niedermolekulare, aliphatische, konstitutionell der Corticosteroidseitenkette ähnliche Verbindungen, wie Dioxyaceton usw., die Linolsäureoxydation katalysieren können⁸. Allerdings besteht zwischen diesen und den Corticosteroiden ein grundsätzlicher Unterschied. Die niedermolekularen, bisher ge-

¹ W. SCHULER und R. MEIER, Hoppe-Seylers Z. 302, 236 (1955).
² W. SCHULER, unveröffentlichte Befunde.
³ F. BERNHEIM, M. K. WILBUR und C. B. KENASTON, Arch. Biochem. Biophys. 38, 177 (1952). – A. OTTOLENGHI, F. BERNHEIM und K. M. WILBUR, Arch. Biochem. Biophys. 56, 157 (1955).
⁴ C. W. SHUSTER, Proc. Soc. exp. Biol. Med. N.Y. 90, 423 (1955).
⁵ W. D. FISHER und K. M. WILBUR, J. Elisha Mitchell Soc. 70, 124 (1954).
⁶ K. M. WILBUR, G. B. KENASTON, N. WOLFSON, A. OTTOLENGHI und M. E. GAULDEN, Anat. Rec. 120, 708 (1954).
⁷ W. SCHULER und R. MEIER, Arch. exp. Path. Pharmacol. 228, 474 (1956).
⁸ W. SCHULER und R. MEIER, Helv. physiol. Acta 15, 279 (1957).

prüften Substanzen sind autoxydabel, und der Verlauf ihrer Wirkung entspricht nicht völlig dem der Cortison-katalyse.

Es schien uns von erheblichem Interesse zu versuchen, einfachste Strukturmodelle aufzufinden, welche die charakteristische katalytische Eigenschaft von Cortison aufweisen, ohne selbst autoxydabel zu sein. In dieser Absicht haben wir unter anderen verschiedene ω -Hydroxy-acetophenonderivate, welche uns von Herrn Prof. REICHSTEIN⁹ zur Verfügung gestellt worden sind, hinsichtlich ihrer katalytischen Wirkung auf die Linolsäureoxydation geprüft, und zwar:

ω -Hydroxy-acetophenon:			
	Substituent:	4	3
ω , 3, 4-Trihydroxy-	acetophenon-OH	-OH	18813/E
ω , 3-Dihydroxy-4-methoxy-acetophenon-	OCH ₃ -OH		17977/E
ω , 4-Dihydroxy-3-methoxy-acetophenon-	OCH ₃	-OCH ₃	14601/E
ω -Hydroxy-3, 4-dimethoxy-acetophenon-	OCH ₃ -OCH ₃		18815/E
ω , 4-Dihydroxy-	acetophenon-OH	-H	18647/E
ω -Hydroxy-3-methoxy-4-acetoxy-acetophenon	-OCOCH ₃ -OCH ₃		18646/E

Methode. Die Linolsäureoxydation wurde, wie beschrieben¹, an Hand der Sauerstoffaufnahme nach WARBURG bestimmt. Ges. Flk. Vol. 2 ml, O₂ als Gas, 37°C, 20 min Temperatenausgleich, stündliche Ablesung, Versuchsdauer 4 h, Substrat 5 mg/ml Linolsäure (Hoffmann-La Roche, Basel). Katalysator: *m*/720 (analog Cortison). Puffer: 0,5 *m*. Phosphat pH 7,0 mit 1% Tween. Leerversuche: Linolsäure allein, Cortison allein, Katalysator allein und Temperaturkontrolle.

Ergebnisse. Die katalytische Wirkung der verschiedenen Acetophenonderivate auf den 4-h-Sauerstoffverbrauch der Linolsäure unter den genannten Bedingungen ist in folgender Tabelle in Prozenten des gleichzeitig bestimmten Sauerstoffverbrauchs der Linolsäure mit äquimolarer Menge Cortison in der Reihenfolge der katalytischen Wirksamkeit angegeben:

Katalytische Wirkung verschiedener ω -Hydroxy-acetophenonderivate			
Katalysator <i>m</i> /720	Substituenten		Prozentualer O ₂ -Verbrauch in 4 h (Cortison = 100%)
	in 4	in 3	
18813/E	-OH	-OH	0
14601/E	-OH	-OCH ₃	etwa 10
17977/E	-OCH ₃	-OH	etwa 20
18647/E	-OH	-H	etwa 30
18646/E	-OCOCH ₃	-OCH ₃	etwa 30
18815/E	-OCH ₃	-OCH ₃	100
—	ω -Hydroxy-acetophenon		140

Während somit die meisten der geprüften Acetophenonderivate nur sehr geringe katalytische Wirkung auf die Linolsäureautoxydation zeigen, katalysiert ω -Hydroxy-acetophenon Linolsäure stärker als Cortison und entspricht das ω -Hydroxy-3, 4-dimethoxy-acetophenon in äquimolarer Konzentration quantitativ dem Cortison. Auch der zeitliche Verlauf der Katalyse mit diesem Derivat entspricht völlig dem mit Cortison. Komplexon III hemmt unter unseren Versuchsbedingungen die Oxydationskatalyse mit diesem Derivate zu 50% bei der

⁹ Herrn Professor Dr. T. REICHSTEIN danken wir auch an dieser Stelle bestens.

gleichen E. K. ($1,6 \times 10^{-7}$) wie die mit Cortison ($2,5 \times 10^{-7}$).

Diskussion. Das ω -Hydroxy-acetophenon katalysiert die Linolsäureautoxydation stärker als Cortison, während ω -Hydroxy-3, 4-dimethoxy-acetophenon quantitativ und qualitativ gleich wie Cortison katalysiert. Das im Ring substituierte Derivat unterscheidet sich von den übrigen nicht oder kaum katalytisch wirksamen Acetophenonderivaten hinsichtlich Ringsubstituenten dadurch, dass es in 3, 4-Stellung zwei Methoxygruppen und weder eine freie noch eine acetylveresterte Hydroxygruppe trägt. Offensichtlich vermag bereits ein derartiger Ringsubstituent die katalytische Wirkung völlig oder nahezu völlig aufzuheben, trotzdem die eigentliche katalytische Wirkgruppe, die Seitenkette, unverändert ist. Der Befund, dass komplexbildungsfähige Substituenten im Ring die katalytische Aktivität der intakten, eigentlichen Wirkgruppe derart stark zu beeinflussen vermögen, erscheint uns wichtig, denn dies könnte eine Erklärung sein, warum dem Cortison verwandte Corticosteroide, trotz gleicher Seitenkette, katalytisch verschiedenen wirksam sind⁷.

W. SCHULER und R. MEIER

Forschungslaboratorien der CIBA Aktiengesellschaft, Basel, Pharmazeutische Abteilung, 16. Mai 1958.

Summary

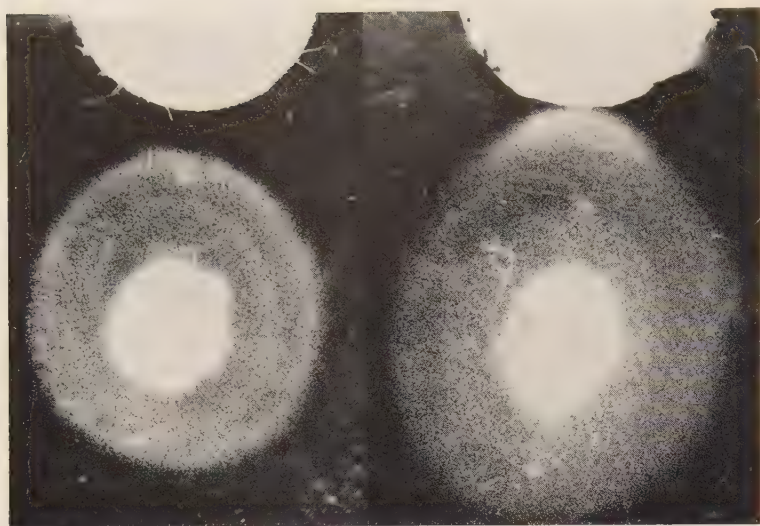
Acetophenone derivatives with a cortisone-like side chain have been investigated for their catalytic effect on the autoxydation of linolenic acid. ω -Hydroxy-acetophenone and the substituted ω -hydroxy-3, 4-dimethoxy-acetophenone are the substances with the simplest structure having a catalytic effect quantitatively and qualitatively similar to that of cortisone.

The significance of substituents in the ring for the catalytic activity of the side chain is discussed.

Die Wirkung der Lipoid- und Polysaccharid-Komponente endotoxischer Lipopolysaccharide gram-negativer Bakterien auf die Leukozytenkultur

Extrakte aus gramnegativen Bakterien vornehmlich polysaccharidischer Natur wirken auf die feste Leukozytenkultur bis zu hohen Verdünnungen emigrationsfördernd¹. In vielen verschiedenen Testen an höheren Tieren und klinisch am Menschen konnte gezeigt werden, dass die im Leukozyten-Emigrationstest wirksamen Bakterienstoffe die bekannten typischen endotoxischen Manifestationen auslösen. Sie sind unter anderem die wirksamsten bislang bekannten Pyrogene². WESTPHAL *et al.* haben 1952 nachgewiesen, dass es sich dabei nicht um reine Polysaccharide, sondern um Lipopolysaccharide handelt³ und dass die Lipoid-Komponente (Lipoid A⁴) für die Pyro-

¹ R. MEIER und B. SCHÄR, Hoppe-Seyl. Z. 307, 103 (1957).
² E. EICHENBERGER, M. SCHMIDHAUSER-KOPP, H. HURNI, M. FRICISAY und O. WESTPHAL, Schweiz. med. Wschr. 85, 1190, 1213 (1955).
³ O. WESTPHAL, O. LÜDERITZ, E. EICHENBERGER und W. KEIDERLING, Z. Naturf. 7 B, 536 (1952).
⁴ O. WESTPHAL und O. LÜDERITZ, Angew. Chem. 66, 407 (1954).



Leukotaktische Wirkung von Lipoid A aus *E. coli*.
 Oben rechts Filterpapier mit aufgetrocknetem Lipoid A (Lösung 10⁻⁴ in Chloroform),
 links Kontrolle (Chloroform).

Nach diesen Prinzipien wurden aus *Salmonella abortus equi* und *E. coli* (Stamm Kröger, Serogruppe 08) die folgenden Substanzen in hochgereinigter Form dargestellt:

- a) Lipopolysaccharid;
- b) Bakt. Lipoprotein;
- c) Lipokasein;
- d) Lipoidfreies Polysaccharid;
- e) Lipoid A.

genität⁴ und viele weitere endotoxischen Wirkungen verantwortlich ist (siehe zum Beispiel auch ⁵). Auf Grund der nachstehend beschriebenen Versuche ergibt sich, dass die Lipoidkomponente der bakteriellen Lipopolysaccharide auch für die leukozytenemigrationsfördernde Wirkung entscheidend ist.

Material und Methodik. Die biologische Untersuchung des isolierten Lipoids A bietet insofern Schwierigkeiten, als das hochgereinigte Material in Wasser nahezu unlöslich ist. Wir haben daher die schon früher benutzte Technik⁴ (vgl. ⁶) der Umkuppelung angewandt: das *Lipopolysaccharid* wurde an ein inertes Protein – im vorliegenden Fall reines, pyrogenfreies Kasein-Hammarsten – gekuppelt (vgl. ⁷) und der künstliche Komplex mit verdünnter Säure behandelt, derart, dass das Polysaccharid quantitativ abgespalten wurde, während Lipoid A am Protein gebunden blieb⁸. In dem so gewonnenen wasserlöslichen, künstlichen *Lipokasein* ist nurmehr Lipoid A bakteriellen Ursprungs; Kasein fungiert als lyphiler Träger. Mit Hilfe des Phenol/Wasser-Verfahrens⁹ können Bakterien so aufgearbeitet werden, dass die endotoxisch wirksame Lipoidkomponente am Bakterienprotein gebunden bleibt¹⁰, wobei stark pyrogene bakterielle *Lipoproteine* (conjugated protein) erhalten werden, die kein Polysaccharid enthalten. Reine *Polysaccharide* aus den Endotoxinen gramnegativer Bakterien sind nur als sogenannte «degraded polysaccharides» im Sinne von MORGAN¹¹ mit Molekulargewichten in der Größenordnung von 20000 bis 30000 darstellbar, weil bei der hydrolytischen Abspaltung des Lipoids A die hochmolekularen Lipopolysaccharide in kleinere Untereinheiten zerfallen, die im genuinen Material durch lipoidale Gruppen zusammengehalten werden.

Bei der Prüfung der *Emigrationsförderung an der Hühnerleukozytenkultur* hielten wir uns an die Angaben von MEIER, DESAULLES und SCHÄR¹². Die Mutterstücke der Kultur wurden vor der Bebrütung während 30 min in mindestens 3 Standard-Verdünnungen der wasserlöslichen Präparate a bis d gebadet. Die Kulturen wurden nach 12stündiger Inkubation bei 37°C photographiert und die Wachstumszonen planimetriert. Für jeden Wert wurden 10–20 Kulturen ausgemessen.

Zur Prüfung der leukotaktischen Wirkung von Lipoid A wurden kreisrunde Filtrierpapierstückchen von 5 mm Durchmesser in eine Lösung von Lipoid A in reinem Chloroform eingelegt und nach sorgfältigem Trocknen in einer Entfernung von etwa 3 mm vom Mutterstück auf den Nährboden gebracht.

Emigrationswirkung verschiedener Komponenten bakterieller Endotoxine

	<i>S. abortus equi</i>			<i>E. coli</i> 08		
	10 ⁻⁶	10 ⁻⁹	10 ⁻¹²	10 ⁻⁶	10 ⁻⁹	10 ⁻¹²
Lipopolysaccharid	+	+	(+)	+	+	+
Bakt. Lipoprotein	+	0	0	+	(+)	0
Lipokasein	+	0	0	+	+	0
Lipoidfreies Polysaccharid	0	0	0	0	0	0
Lipoid A	leukotaktisch			leukotaktisch		
Kasein	0	0	0			

+ = signifikant vergrößerter Auswanderungshof;
 (+) = nicht signifikant vergrößerter Auswanderungshof;
 0 = ohne Wirkung.

Ergebnisse und Diskussion. Die in der Tabelle zusammengestellten Resultate zeigen erhebliche quantitative Unterschiede zwischen den einzelnen Präparaten. Alle diejenigen, welche Lipoid A enthalten, wirken mehr oder weniger stark emigrationsfördernd, während das lipoidfreie Polysaccharid beider Keime ohne Einfluss auf die Leukozytenkultur ist. Reines Lipoid A wirkt deutlich leukotaktisch (siehe Abbildung). Diese Wirkung ist mit Sicherheit nicht auf etwa vorhandene Spuren begleitenden Lipopolysaccharids zurückzuführen, was durch die Art

⁵ J. G. HOWARD, D. ROWLEY und A. C. WARDLAW, *Nature* 179, 311 (1957).
⁶ Y. TAKEDA, N. KASAI, M. ARAKI und O. T. ODAKA, *Hoppe-Seyl. Z.* 307, 49 (1957).
⁷ O. LÜDERITZ, O. WESTPHAL, E. EICHENBERGER und E. NETER, *Biochem. Z.* 330, 21 (1958).
⁸ F. BINKLEY, W. F. GOEBEL und E. PERLMAN, *J. exp. Med.* 81, 331 (1945).
⁹ O. WESTPHAL, O. LÜDERITZ und F. BISTER, *Z. Naturf.* 7B, 148 (1952).
¹⁰ O. WESTPHAL, O. LÜDERITZ und W. KEIDERLING, *Zbl. Bakt. I. Orig.* 158, 152 (1952).
¹¹ W. T. J. MORGAN und S. M. PARTRIDGE, *Biochem. J.* 34, 169 (1940); 35, 1140 (1941); *Brit. J. exp. Path.* 23, 151 (1942).

¹² R. MEIER, P. A. DESAULLES und B. SCHÄR, *Arch. exp. Path. Pharmac.* 224, 104 (1955).

der Reinigung von Lipoid A ausgeschlossen ist. Die quantitativen Unterschiede können nicht auf einen wechselnden Lipoidgehalt der einzelnen Präparate zurückgeführt werden, sondern sind offenbar durch die physikalisch-chemischen Eigenschaften der Trägersubstanzen mitbedingt. Polysaccharide als lyophile Träger scheinen in dieser Beziehung besonders günstig, wie wir auch am Beispiel anderer biologischer Wirkungen (Pyrogenität u.a.) zeigen konnten.

Die durchweg etwas geringere Wirksamkeit der Präparate aus *S. abortus equi* gegenüber denjenigen aus *E. coli* 08 stimmt mit unserer Erfahrung überein, wonach das Lipoid A aus *S. abortus equi* gegen Hydrolyse und andere chemische Einflüsse weniger stabil ist als dasjenige aus *E. coli*.

Der Polysaccharidanteil in den bakteriellen Lipopolysacchariden vermittelt die Wasserlöslichkeit der Präparate und damit den hohen Dispersionszustand des Lipoids A in Lösung; das Polysaccharid ist überdies Träger der immunspezifischen Eigenschaften (O-Antigen) der betreffenden Bakterienart¹³. Demgegenüber enthält der Lipoid-Anteil (Lipoid A) die für die endotoxischen Wirkungen wesentlichen Wirkgruppen¹⁴. Auf Grund der vorliegenden Versuche über die leukotaktische Wirkung der bakteriellen Lipopolysaccharide kann geschlossen werden, dass hierfür ebenfalls Wirkgruppen im jeweiligen Lipoid-A-Anteil wesentlich sind.

GISELA SCHMIDT, E. EICHENBERGER
und O. WESTPHAL

Dr.-A.-Wander-Forschungsinstitute, Bern und Freiburg-Zähringen, den 8. April 1958.

Summary

Highly purified lipopolysaccharides derived from gram-negative bacteria (endotoxins) stimulate the migration of leucocytes from cultures of chick leucocytes. This effect, as well as many endotoxic manifestations of the preparations *in vivo* (pyrogenicity, toxicity, etc.), is induced by their lipid fraction (lipid A), while the respective species-specific polysaccharide functions as lyophilizing carrier which, fundamentally, can be substituted by other inert carriers (e.g. protein).

¹³ O. WESTPHAL und O. LÜDERITZ, Angew. Chemie 66, 407 (1954). – Siehe zum Beispiel A. M. STAUB und R. TINELLI, Bull. Soc. Chim. biol., Paris 39, Suppl. I, 65 (1957).

¹⁴ E. NETER, O. WESTPHAL, O. LÜDERITZ, E. A. GORZYNSKI und E. EICHENBERGER, J. Immunol. 76, 377 (1956). – O. WESTPHAL, O. LÜDERITZ, E. EICHENBERGER und E. NETER in Chemistry and Biology of Mucopolysaccharides, Ciba Found. Symp. (J. & A. Churchill Ltd., London 1958), p. 187.

Exudative Diathesis Produced by Vitamin E-Deficient Diets Without Polyenoic Fatty Acids

In earlier studies (DAM, KOFOED NIELSEN, PRANGE, and SØNDERGAARD¹) we have examined the content of polyenoic fatty acids in two kinds of yeast, viz. Torula 3N and Fleischmann 50B, used as the source of protein in vitamin E-deficient diets for chicks. Torula 3N, which gave rise to exudates, contained 5.3% total fatty acids

with 44.7% dienoic and 2.6% trienoic, whereas Fleischmann 50B, which did not produce exudates, contained almost no polyenoic fatty acids. Torula yeast extracted with alcohol still contained 2.7% total fatty acids with 45.3% dienoic and 3.6% trienoic¹, and gave rise to exudates (BIERI, POLLARD, and BRIGGS²).

Therefore, in analogy with previous experiences with casein-cod liver oil diets, it seems reasonable to assume that, also in the experiments with Torula yeast, the polyenoic fatty acids are an important factor in the development of exudative diathesis.

In a search for possible additional factors, we have examined a series of different yeasts with respect to their fat content and ability to produce exudative diathesis in chicks. Among these yeasts we have found one which is of particular interest, because, as in Fleischmann yeast 50B, the content of polyenoic fatty acids is practically nil, and nevertheless it produces exudates in chicks when fed without vitamin E. This yeast is a dried *Saccharomyces cerevisiae* obtained from The Distillers Company Ltd., London under the designation 'Flake Yeast'. It contained 4.4% total fatty acids and 2.0% unsaponifiable matter. The content of dienoic fatty acids in the total fatty acids was 0.6%. The amounts of trienoic, tetraenoic, pentaenoic, and hexaenoic acids were nil. No significant amount of trans fatty acids could be detected by means of infrared spectrophotometry.

Composition of basal diets, g per 100 g

Flake yeast	40.00	60.00
Gelatine	3.00	3.00
Salt mixture ³	5.17	5.17
Vitamin mixture ³	0.10	0.10
Choline chloride	0.20	0.20
Sucrose	51.53	31.53

Plus 0.001 g dicalcium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid ester (Synkavit 'Roche') per 100 g diet.

Vitamins A and D₃ were given in aqueous solution⁴, 0.1 ml twice a week.

When fed at a 40%, resp. 60% level in a diet of the composition indicated in the Table, 'Flake yeast' caused exudates in 9, resp. 5 out of 10 chicks within 29 days. In a repetition of the experiment, 8, resp. 6 out of 10 chicks showed exudates within 33 days. These results suggest that 'Flake yeast' contains an insufficient amount of a protective factor. The exudates were less severe than those produced by corresponding Torula yeast diets.

One more peculiarity was noted in the feeding experiments with 'Flake yeast'. Fat tissue in which exudate had occurred contained, in 19 out of 23 cases, no peroxide detectable by the method of KING, ROSCHEN, and IRWIN⁵, modified by DAM and GRANADOS⁶.

This might indicate that the *in vivo* peroxidation of body fat usually observed in connection with exudative diathesis produced by casein-cod liver oil diets and (to a

² J. G. BIERI, C. J. POLLARD, and G. M. BRIGGS, Fed. Proc. 16, 381 (1957).

³ H. DAM and E. SØNDERGAARD, Acta pharm. tox., Kbh. 9, 131 (1953).

⁴ H. DAM, S. HARTMANN, J. E. JACOBSEN, and E. SØNDERGAARD, Acta physiol. scand. 41, 149 (1957), Table 2.

⁵ A. E. KING, H. L. ROSCHEN, and W. H. IRWIN, Oil & Soap 10, 105 (1933).

⁶ H. DAM and H. GRANADOS, Acta physiol. scand. 10, 162 (1945).

¹ H. DAM, G. KOFOED NIELSEN, I. PRANGE, and E. SØNDERGAARD, Exper. 13, 493 (1957).

lesser degree) by *Torula* yeast diets is not *per se* the cause of the exudation process but a sign of the disappearance of the antioxidant vitamin E from tissue simultaneously containing a certain amount of polyenoic fatty acids. Further, it is possible that the hematin compounds released in the hemorrhagic phase of the exudation process accelerate an existing tendency to peroxidation (cf. TAPPEL⁷).

These considerations may also serve to explain the observation that selenium dioxide prevents peroxidation in chicks fed vitamin E-free *Torula* yeast diets: by preventing exudates, selenium will slow down the appearance of peroxides otherwise accelerated through the hemorrhagic phase of the exudation process.

Selenium dioxide does not counteract the *in vitro* autoxidation of cod liver oil incorporated in casein diets, nor does it prevent brown coloration and peroxidation of depot fat in rats fed vitamin E-free cod liver oil-casein diets⁸. It is therefore unlikely that the protective action of selenium dioxide against the exudative diathesis in chicks is due to an *in vivo* antioxidant effect.

H. DAM, G. KOFOED NIELSEN,
I. PRANGE, and E. SØNDERGAARD

Department of Biochemistry and Nutrition, Polytechnic Institute, Copenhagen, April 29, 1958.

Zusammenfassung

Bei Fütterung von Küken mit einer Vitamin-E-freien Nahrung, die als Eiweissquelle eine spezielle Trockenhefe, frei von mehrfach ungesättigten Fettsäuren, enthielt, wurde eine jedoch verhältnismässig milde, exsudative Diathese beobachtet.

⁷ A. L. TAPPEL, Arch. Biochem. Biophys. 44, 378 (1953); J. biol. Chem. 217, 721 (1955).

⁸ Paper in preparation.

Isolation of a Toxic Fraction from Uraemic Blood

In our former studies¹, it was observed that uraemic blood streaming through Dovex 50, Dovex 2, and IR 4B ion-exchange resins would become toxic. The fact that similarly treated control blood samples did not become toxic led us to the assumption that uraemic blood might contain some preformed toxin.

In the present experiment, we aimed at producing this substance in a more concentrated form.

Methods. Dogs were subjected to bilateral nephrectomy under sterile conditions. 72–96 h postoperatively the animals were exsanguinated and the cerebrospinal fluid was obtained through cisternal puncture. The toxicity tests were performed on rats and mice.

15 nephrectomized and 8 control dogs and 35 human blood specimens (uraemic or of diseases of non-renal origin), as well as the cerebrospinal fluid of 14 human subjects and of 5 dogs, were used in the course of the experiment.

Experimental. For purifying the toxin, the acid alcoholic fraction has so far proved to be the most effective.

¹ I. DÉSI, I. FEHÉR, P. WEISZ, and E. SZOLD, Z. ges. inn. Med. 24, 1127 (1957).

Kind of experiment	Number of experiments	Average of NPN mg%	Number of animals used	Number of animals that died
Uraemic blood . .	28	99 (53.5–195)	mice 19 rats 63	17 58
Control blood . .	30	36 (28.8–48)	mice 17 rats 27	0 0
Uraemic cerebrospinal fluid . . .	5	150 (128–174)	rats 7	7
Control cerebrospinal fluid . . .	14	20 (6–42)	rats 14	0

100 ml of plasma centrifuged until pure were dialyzed at a temperature of about + 5°C in cellophane bag in flowing water. Aethyl alcohol was added until a 50% concentration and acetic until a 1% final concentration was achieved. The dense substance full of precipitation was then placed in a hot-water bath for 10 min and centrifuged. The deposit was twice washed in 25 ml of 75% alcohol and the precipitation was discarded. The washing fluid and the supernatant were mixed and condensed in vacuum from the water bath to $\frac{1}{6}$ of the original volume. 0.08 g of NaHCO₃ and 50 ml of 70% alcohol were added to the residue, whereafter the substance was repeatedly centrifuged and washed as before. The mixed fluids were then evaporated from water bath to achieve the $\frac{1}{10}$ of the original volume. The evaporation was performed in vacuum. The substance was shaken out with a small quantity of charcoal (50–80 mg/10 ml of the substance) and the sediment was cast off. The substance thus obtained was used in the further experiments.

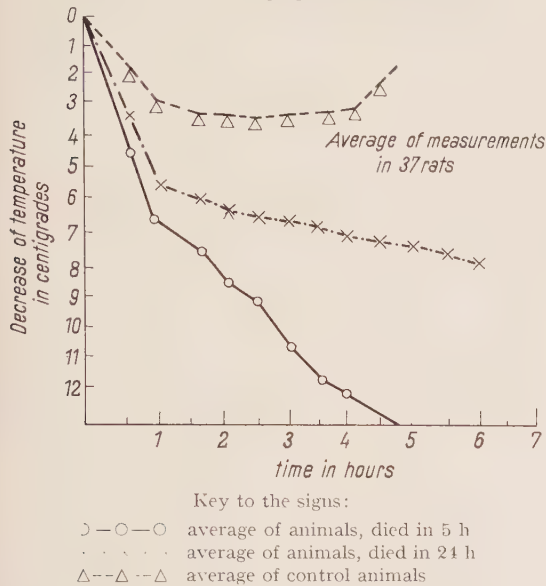
The K, Na, Cl, P, protein- and non-protein nitrogen of preparates thus obtained, showed no difference whether originating from the serum of uraemic or control dogs. According to the administered dose, the toxin caused various symptoms in 50–80 g rats. The toxin was injected intraperitoneally. 1–1.5 ml of the substance would bring about death of the animals with violent tonic spasms and dyspnoe within 30 min. We suppose respiratory paralysis to be the immediate cause of death. The administration of a smaller dose killed the animals within 5 h, while a still smaller dose resulted in death within 24 h. The administration of a medium dose produced the first symptoms after 15–30 min. The spontaneous activity of the animal was reduced and later on disappeared. First there is a response to the sensation of pain, the animal trying to run away, but the movements are incoordinated, the gait is unsteady with a tendency to fall; when it tries to run again, it rolls about a few times and falls again after a few steps. Exhaustion takes place very soon and the animal shows no response to repeated stimuli, becoming completely atonic after a short while. There is no more sensation of pain, it remains still and completely atonic. Respiration becomes shallow and overhasty and the whole condition is interrupted by paroxysms of tonic convulsions in the midst of which the animal dies (Table).

The blood pressure of the intoxicated animals was taken in 10 cases, but no reduction was shown even in the end state.

A correlation was observed between the amount of toxin injected and the variations in the rectal temperature of the rats. The measurements were carried out under conditions corresponding to 18–20°C room temperature. There was a 10° reduction in the temperature within 2 h due to large doses. There was a 3°C reduction of temperature also after the administration of control plasma, but

a few hours later the animals regained their original temperature and never died (Figure).

Changes in the rectal temperature of rats after injecting uraemic and control preparates



Toxin obtained from the blood and cerebrospinal fluid of uraemic patients produced the same symptoms. Preparates obtained from healthy individuals, or of others suffering from any disease of non-renal origin, failed to produce these symptoms.

Since it is wellknown that hypothermy is one of the clinical symptoms of human uraemia², we tested whether this phenomenon was demonstrable in animals as well. After bilateral nephrectomy of 45 rats, we found that the temperature showed a gradual reduction between the operation and the death of the animals, reaching a level of about 25°C.

The preparate will become inactive if dialyzed in flowing water in a cellophane bag suggesting that the toxin is a substance of small molecules. Since the native uraemic plasma has no toxic effect, but would become effective if dialyzed and prepared in the manner described above, we concluded that the toxin may circulate in the uraemic blood in the form bound to protein or to some other large molecule without being active in that state.

Incubated with pancreatic suspension or trypsin (Merck), the toxin would become inactive, suggesting that our substance may be a peptide.

Since, as a rule, the animals died after a severe drop in temperature, we tried to avert the action of toxin by the administration of a pyrogen agent. 1 h after receiving the toxin, the animals were injected intraperitoneally with 20 mg/kg body weight of Amphetamine. After this dose, the reduction in temperature discontinued, but the animals still died, even earlier than those treated only with the toxin.

Summarizing the results, it may be stated that we succeeded in demonstrating from blood samples of uraemic individuals and dogs, and from their cerebrospinal fluid, a substance as yet unknown as far as we know, which might play a role in the production of uraemic symptoms.

² E. LAUDA, *Lehrbuch der inneren Medizin*, vol. 3 (Springer, Wien 1951), p. 223.

Judging by the experiments carried out so far, the substance is considered to be a peptide of small molecules.

I. FEHÉR, I. DÉSI, and E. SZOLD

Pathophysiological Institute and the Urological Clinic of the Medical University, Budapest (Hungary), May 3, 1958.

Zusammenfassung

Aus urämischem Menschen- und Hundeblood und Liquor isolierten wir eine uns bisher unbekannte Substanz, die unserer Ansicht nach für die Entstehung von urämischen Symptomen mitverantwortlich sein dürfte. Auf Grund unserer bisherigen Untersuchungen halten wir die Substanz für ein kleinemolekulares Peptid.

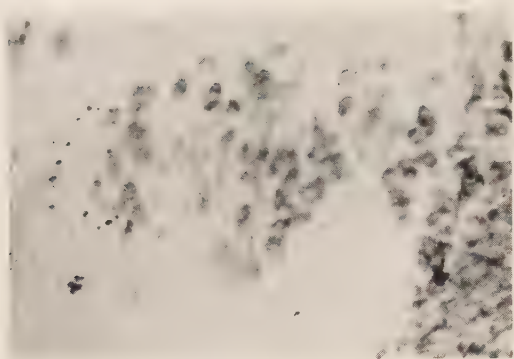
Acetylcholinesterase, Acid Phosphatase, and Succinic Dehydrogenase in the Hypothalamic Magnocellular Nuclei after Chlorpromazine Administration

Histochemical studies

Strong enzyme activity has been found to exist in the magnocellular nuclei of the hypothalamus, where neurosecretory material is formed¹; the following enzymes have been demonstrated histochemically: acetylcholinesterase², acid phosphatase³, and succinic dehydrogenase⁴. The influence of chlorpromazine upon the organism is primarily due to its depressive effect upon the central nervous system in the formatio reticularis of the brain stem and in the hypothalamus of the diencephalon⁵. We have previously investigated the influence of chlorpromazine upon the neurosecretory substance of the hypothalamic magnocellular nuclei and upon the secretion of anti-diuretic hormone, and we found that chlorpromazine depresses the hypothalamus⁶. The purpose of the present study is to determine to what extent chlorpromazine affects the histochemically demonstrable acetylcholinesterase, acid phosphatase, and succinic dehydrogenase activity in the supraoptic and paraventricular nuclei of the hypothalamus.

Chlorpromazine (Largactil, May & Baker) was given subcutaneously, 25 mg/kg body weight, to six adult female albino rats for ten days. The control group consisted of an equal number of animals. The subjects were killed by rapid decapitation 4 h after the last injection. From their hypothalamus the acetylcholinesterase was determined according to KOELLE's modification⁷, the acid phosphatase according to ERÄNKÖ⁸, and the succinic dehydrogenase according to SELIGMAN and RUTENBURG⁹.

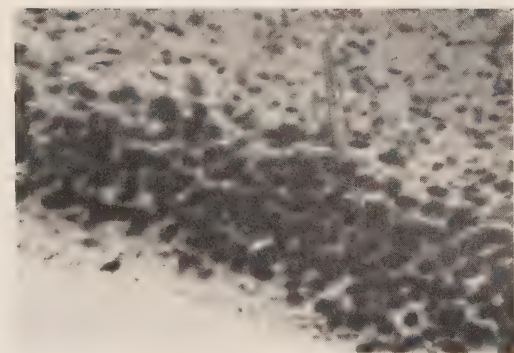
¹ E. SCHARRER, *Exper.* 10, 264 (1954).
² V. C. ABRAHAM, G. B. KOELLE, and P. SMART, *J. Physiol.* 139, 137 (1957).
³ O. ERÄNKÖ, *Acta physiol. scand.* 24, 1 (1951).
⁴ N. SHIMIZU and N. MORIKAWA, *J. Histochem. Cytochem.* 5, 334 (1957).
⁵ R. S. COURVOISIER, J. FOURNEL, R. DUCROT, M. KOSLKY, and P. KOETSCHUT, *Arch. int. Pharmacodyn.* 92, 305 (1953). – H. E. HIMWICH and F. RINALDI, *Brain Mechanism and Drug Action* (Springfield 1957).
⁶ E. KIVALO, U. K. RINNE, and P. MARJANEN, *Ann. med. exp. biol. Fenn.*, in press (1958).
⁷ G. B. KOELLE, *J. Pharmacol.* 114, 167 (1955).
⁸ O. ERÄNKÖ, *Ann. med. exp. biol. Fenn.* 29, 287 (1951).
⁹ A. M. SELIGMAN and A. M. RUTENBURG, *Science* 113, 317 (1951).



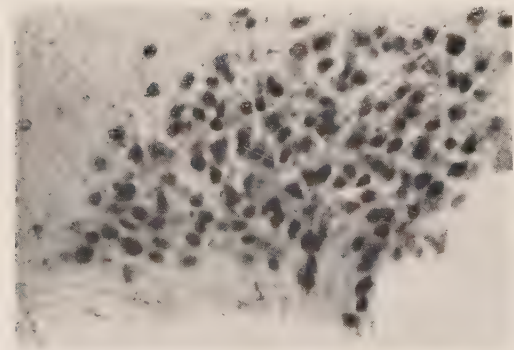
A



B



C



D

A Distribution of acetylcholinesterase in the nucleus supraopticus of control rat. B The same after administration of chlorpromazine. C Acid phosphatase activity in the nucleus supraopticus of control rat. D The same after chlorpromazine treatment. All 180

The normal acetylcholinesterase and acid phosphatase of the hypothalamic supraoptic and paraventricular nuclei were confirmed to be in accordance with earlier results, whereas the succinic dehydrogenase activity is slightly stronger in our opinion, than is shown by the earlier investigations. On comparison between the acetylcholinesterase, acid phosphatase and succinic dehydrogenase activities in the said nuclei of the animals under chlorpromazine administration and the controls, a moderate reduction in activity was observed for each enzyme with the animals that had been given chlorpromazine.

Our results are in agreement with our previous investigation, in which we showed that chlorpromazine lowers the discharge of antidiuretic hormone from the supraoptic nucleus and the paraventricular nucleus⁶. They are also in agreement with biochemical investigations, which have shown that chlorpromazine generally depressed the enzyme system in the brain¹⁰. It is further conceivable that the reduced activity of these various enzyme systems is connected with the sedative effects caused by chlorpromazine.

E. KIVALO*, U. K. RINNE*, and S. MÄKELÄ

Department of Anatomy, University of Turku (Finland), June 2, 1958.

Zusammenfassung

Der Einfluss des Chlorpromazins auf die Acetylcholinesterase, saure Phosphatase- und Bernsteinsäuredehydrogenaseaktivität des Nucleus supraopticus und Nucleus paraventricularis wurde histochemisch untersucht. Es wurde festgestellt, dass Chlorpromazin bei sämtlichen Enzymen eine mässige Herabsetzung der Aktivität bewirkt. Die Vermutung wird ausgesprochen, dass mit der Sedativwirkung des Chlorpromazins eine Verminderung der Enzymaktivität verknüpft ist.

¹⁰ J. BERNSOHN, I. NAMAJUSKA, and B. BOSSES, *J. Neurochemistry* 1, 145 (1956).

* Aided by a grant from the Sigrid Jusélius Stiftelse.

Über das Glucosid von 1,2-Diphenyl-3,5-dioxo-4-*n*-butyl-pyrazolidin (Phenylbutazon)

Von einer Reihe therapeutisch wichtiger Glykoside, die schon lange unseren Arzneischatz bereichern, ist bekannt, dass das Aglykon allein eine zum Teil viel schwächere Wirkung als das Glykosid besitzt. Andererseits sind zum Beispiel von dem als Tuberkulostaticum bekannten Isonikotinsäurehydrazid-Hydrazone mit Aldosen bekannt, die sich durch eine bessere Verträglichkeit, das heisst grössere therapeutische Breite auszeichnen¹. Ausserdem ist es möglich, mit solchen Derivaten die Resorption zu verändern und so eventuell eine protrahierte Wirkung zu erzielen². Da Phenylbutazon als β -Dicarbonylverbindung leicht enolisiert, erschien es uns interessant, das Enolglucosid darzustellen. Von BALLOU und LINK³ sind eine

¹ H. LÜDHOF und E. KRÖGER, *Klin. Wschr.* 34, 451 (1956). – G. BROUET, B. N. HALPERN, J. MARCHE und J. MALLET, *Presse méd.* 61, 863 (1953). – J. KIMMIG, F. KRÜGER und J. MEYER-TOHN, *Arzneimittelforsch.* 7, 157 (1957).

² CH. A. COLWELL, A. R. HESS, C. J. WOODS und E. J. DES AUTELS, *J. Lab. clin. Med.* 46, 597 (1955).

³ C. E. BALLOU und K. P. LINK, *J. Amer. chem. Soc.* 72, 3147 (1950); 73, 1134 (1951).

Reihe solcher Glucoside mit Acetessigester und ähnlichen Verbindungen dargestellt worden. Nach derselben Methode erhielten wir aus Acetobromglucose⁴ und Phenylbutazon 1- β [1',2'-Diphenyl-3'-keto-4'-*n*-butyl-pyrazol-5'-]-2,3,4,6-tetraacetyl- β -glucosid (I) in einer Ausbeute von 32,5%. Aus Essigester/Äther F. 155–156°. $[\alpha]_D^{20} = -44^\circ$ (in CHCl_3 ; $C = 0,51$). Die Abspaltung der Acetylgruppen nach ZEMPLÉN oder mit NH_3 in der Kälte gelang erwartungsgemäss infolge der Alkaliempfindlichkeit des Glucosides nicht. Kürzlich wurde nun von BREDERECK *et al.* am Beispiel der β -Pentaacetylglucose und des Tetraacetyl- α -methylglucosids die Acylspaltung mit Diazomethan beschrieben⁵. Wir erhielten bei der Einwirkung einer Diazomethanolösung auf I nach obiger Methode neben unverändertem Ausgangsmaterial eine Verbindung vom Schmelzpunkt 85–86° (aus Methanol) in einer Ausbeute von 65%, deren Summenformel nach der Analyse gut mit dem Methylenoläther des Phenylbutazons übereinstimmte.

Diese Struktur wird durch die Absorptionsspektren im UV.- (Max. 250 μ , $\log \epsilon = 4,22$ in Methanol) und IR.-Gebiet ($\nu \text{ C-O } 5,95 \mu$, $\nu \text{ C=C } 6,13 \mu$, CH_2Cl_2 -Lösung) bestätigt⁶.

Wir untersuchten noch die Resorption und Verteilung des tetraacetylierten Glucosides des Phenylbutazons bei der Ratte, dem Kaninchen und beim Menschen⁷, da wir infolge der leichten Spaltbarkeit der Verbindung angenommen haben, dass eine Verseifung im Magen bzw. Darm stattfinden würde. Die D.l. 50, *per os*, von I bei der Maus lag über 5000 mg/kg. Da der prozentuale Anteil des glykosidisch gebundenen Phenylbutazons bei I etwa 50% beträgt, wurden, um vergleichbare Daten über die orale Resorption zu erhalten, die doppelten Dosen an I verabreicht. Die Bestimmungen des Phenylbutazons wurden nach BURNS *et al.*⁸ ausgeführt. Damit festgestellt werden konnte, ob im Plasma noch die intakte Glucosid-Bindung vorliegt, wurden parallele Analysenproben vor der Bestimmung einer sauren Hydrolyse unterworfen.

Interessanterweise wurde nun gefunden, dass beim Menschen bei einer Dosis von 1 g oral und bei der Ratte (0,2 g/kg oral) I praktisch kaum resorbiert wird. Beim Kaninchen hingegen wurde nach 6, 8 und 24 h ein gewisser Plasmaspiegel erreicht, und es konnten auch in Leber, Niere und Herzmuskel Phenylbutazon nachgewiesen werden. Die Werte erreichen jedoch nicht diejenigen des Phenylbutazons allein. Es liegen im übrigen keine Anhaltspunkte dafür vor, dass I als intaktes Molekül, das heisst mit erhaltener glykosidischer Bindung im Blut erscheint.

CH. J. MOREL

Pharmakologische Laboratorien der J. R. Geigy AG., Basel, 29. Mai 1958.

Summary

The synthesis and properties of the acetylated glucoside of phenylbutazon are described. The deacetylation of this enol-glucoside, even under neutral conditions, failed because of the lability of this compound.

⁴ Org. Synth. Col. Vol. 3, 10 (1955).

⁵ H. BREDERECK, R. SIEBER und L. KAMPHENKEL, Chem. Ber. 89, 1169 (1956). – Siehe auch TH. WIELAND und R. H. ROTHHAUPT, Chem. Ber. 89, 1176 (1956).

⁶ Vgl. E. GIROD, R. DELLEY und F. HÄFLIGER, Helv. chim. Acta 40, 408 (1957). – Die IR.-Spektren verdanken wir Herrn Dr. E. GIROD und das UV.-Spektrum dem analytischen Labor (R. DELLAY).

⁷ Herrn Dr. B. HERRMANN sind wir für die Durchführung der Tierversuche sehr zu Dank verpflichtet.

⁸ J. J. BURNS *et al.*, J. Pharmacol. exper. Therap. 109, 346 (1953).

Effect of Colchicin on Poliomyelitis Virus Synthesis and Enzyme Activities of Suspended Rhesus Kidney Fragments

Influence of chemical and physical agents on cell multiplication, virus production, and cellular enzymes was studied to elucidate the mechanism of virus multiplication. Observations with colchicin (Cn), an antimitotic¹, are reported. Maitlandtype of tissue cultures (TC) were used² with synthetic medium 697³ containing various concentrations of the drug⁴. Inoculation with 100 TCPD of Mahoney strain was carried out on the sixth day of incubation at 37°C, the virus harvested on the eleventh. TC were pooled, homogenized then assayed for enzymes and virus⁵. Turbidity readings served for comparison of the tissue mass⁶. Table I (group 1) presents findings on non-infected tissues. Increase of monoesterases and doubling of turbidity values in the former is striking. Infected cultures exhibited a significant decrease in 5-nucleotidase, enhanced alkaline phosphatase and a non-significant decrease in acid phosphatase⁶. The moderate increase in turbidity and the high titres are also of interest. Findings on diesterases are shown in Table II. *Virus yield was good in all TC s.* The smallest amount of Cn did not interfere with RN-ases on the sixth day, but depressed the activities (16–18%) till the eleventh. The virus caused insignificant changes⁷. The acid DN-ase displayed moderate, the alkaline DN-ase high activity on the sixth day. Freezing (–70°C) activated the former markedly⁸; less so the latter one. The acid DN-ase increased, the alkaline decreased on the eleventh day. The effect of freezing was the same as before at pH 5, but it was absent at pH 7. Infection depressed the acid DN-ase⁸, without altering the effect of cold. The opposite was found with alkaline DN-ase, emphasizing the existence of two distinct DN-ases⁹. With larger amounts of Cn, the nuclease activities decreased progressively in non-infected (alkaline DN-ase!) but were enhanced in infected TC. This feature was greatest with alkaline DN-ase. There was a < 10% decrease with acid RN-ase. Doubling the Cn concentration enhanced both nucleases (especially the alkaline ones) on the sixth day; this effect was reversed by further incubation. Infection aggravated these changes in the presence of highly toxic Cn doses². The behaviour of cultures without Cn (both infected and normal controls) has been described². To conclude, we might say that the Cn *did not interfere with virus synthesis* although it affected specific nucleotidase and nuclease-systems¹⁰. A differential influence on the latter is of interest; the DN-ases are apparently more sensitive to it. Optimal Cn concentrations might be necessary for activation or inhibition¹⁰. The

¹ A. F. W. HUGHES, *The mitotic cycle. The cytoplasm and nucleus during interphase and mitosis* (Butterworth, London 1952).

² E. KOVÁCS, Canad. J. Biochem. Physiol. 34, 600 (1956); Biochem. Z. 330, 113 (1958).

³ G. M. HEALY, D. C. FISCHER, and R. C. PARKER, Canad. J. Biochem. Physiol. 32, 327 (1954). – E. KOVÁCS, Canad. J. Biochem. Physiol. 34, 619 (1956).

⁴ Courtesy of Dr. A. H. FRANKLIN of the Department of Bacteriology, University of Toronto, Ont. (Canada).

⁵ E. KOVÁCS, Canad. J. Biochem. Physiol. 34, 600 (1956).

⁶ E. KOVÁCS, J. exper. Med. 104, 589 (1956); Minerva Med. (Torino) 48, 2157 (1957).

⁷ E. KOVÁCS, Proc. Soc. exp. Biol. Med. 92, 183 (1956); G. Malat. Infett. Paras. 10, 190 (1958).

⁸ E. KOVÁCS, Proc. Soc. exp. Biol. Med. 92, 183 (1956); G. Malat. Infett. Paras. 10, 190 (1958); Z. Naturf. 13b, 34 (1958).

⁹ E. KOVÁCS, Canad. J. Biochem. Physiol. 34, 600 (1956); Proc. Soc. exp. Biol. Med. 92, 183 (1956); Z. Naturf. 13b, 34 (1958).

¹⁰ K. LANG, G. SIEBERT, and W. ESTELMAN, Exper. 7, 378 (1951).

Table I
Effect of colchicine and poliomyelitis virus on phosphomonoesterases* of Maitland-Cultures

Homogenates of pools from	Turbidity, Klettunits /ml	Incubation of TC 6 days at 37° C			Turbidity Klettunits /ml	Incubation of TC 11 days at 37° C		
		Acid phos- phatase pH 5	Alkaline phosphatase pH 10.9	5-Nucleo- tidase pH 8.5		Acid phos- phatase pH 5	Alkaline phosphatase pH 10.9	5-Nucleo- tidase pH 8.5
		As increase in inorganic Phospho- rus µg/ml of the system				As increase in inorganic Phospho- rus µg/ml of the system		
3 normal tissue cultures of Rhesus kidney, in 20 ml Medium 697 con- taining 0.05 µg colchicine	80	0.4 µg	1.3 µg	4.0 µg	145	3.0 µg	3.0 µg	13.0 µg
Same as above, after 5 days infec- tion with Type I polio. virus. Final titer: 6.9!	→				100	2.5 µg	4.3 µg	9.4 µg

* Test-system for Monoesterases: 0.5 ml Brei + 9.5 ml buffered substrate. Incubation 22 h at 37°C. For techniques see ref.⁵.

Table II
Effect of colchicine and poliomyelitis virus on nucleases*

Homogenate of pools from	Turbidity Klettunits /ml	Incubation of TC 6 days at 37°C				Turbidity Klettunits /ml	Incubation of TC 11 days at 37°C			
		RN-ase pH 5 pH 7.6 As increase in total acid soluble Phosphorus µg/ml of system		DN-ase pH 5 pH 7 As % decrease of viscosity of system			RN-ase pH 5 pH 7.6 As increase in total acid soluble Phosphorus µg/ml of system		DN-ase pH 5 pH 7.6 As % decrease of viscosity of system	
3 normal tissue cultures of Rhesus kidney in 20 ml Medium 597, con- taining 0.05 µg colchicine	80	10.6	10.0	6.1 24.0 (21.5)+ (28.2)+	135	9.0	8.2	10.0 8.2 (23.0)+ (8.0)+		
As above, after 5 days infection with Type I polio. virus. Final-titer: 6.5!	→				135	8.4	8.0	4.5 14.0 (20.0)+ (11.0)+		
Normal, as above in 20 ml Medium 697, containing 0.5 µg colchicine	70	6.0	5.5	4.5 11.3	135	4.6	5.2	4.0 1.0		
As above, after 5 days infection with Type I polio. virus. Final-titer: 6.9!	→				100	4.2	6.4	13.0 8.0		
Normal, as above in 20 ml Medium 697, containing 1.0 µg colchicine	68	8.0	13.5	7.0 19.5	145	7.2	6.0	6.0 6.0		
As above, after 5 days infection with Type I polio. virus. Final-titer: 6.9!	→				100	6.3	2.3	2.3 3.0		

* Incubation: 2 h at 46°C for RN-ase, 1 h at 30°C for DN-ase. RN-ase system: 0.5 ml Brei + 2 ml 0.1% RNA in Buffer + 1.5 ml Buffer. – DN-ase system: 0.5 ml Brei + 2 ml highly polymerized DNA in water (0.1%) + 2 ml Buffer containing 0.01% Gelatine + 0.025 M MgSO₄ (final concentrations). For techniques see ref.⁵.
† Same tissue cultures assayed after one freezing and thawing.

activation of DN-ascs in TC by cold was discussed previously¹¹; the virus may alter this behaviour and also reverse the effect of non-lethal Cn doses. These findings emphasize that the action of this antimitotic is exerted through enzymes¹². Further studies for correlation of cytological, biological, and biochemical changes are needed on cell cultures.

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Zusammenfassung

Colchicin beeinflusst in antimitotischen und zytotoxischen Dosen die Poliomyelitisvirus-Ausbeute in Maitland-Kulturen von Rhesusaffen-Nieren nicht wesentlich. Die Einwirkung der Droge auf Phosphomonoesterasen, Ribonukleasen und Desoxyribonukleasen derselben Kulturen wird ebenfalls beschrieben.

¹¹ E. Kovács, Z. Naturf. 13b, 34 (1958).
¹² K. LANG, G. SIEBERT, and W. ESTELMAN, Exper. 7, 378 (1951). – E. Kovács, Canad. J. Biochem. Physiol. 34, 600 (1956). – A. F. W. HUGHES, The mitotic cycle. The cytoplasm and nucleus during interphase and mitosis (Butterworth, London 1952).

Evidence of Recruiting Responses in the Cat's Mesencephalic Reticular Formation¹

DEMPSEY and MORISON² first described the production of recruiting responses in the cerebral cortex upon stimulation of certain thalamic nuclei. Their own findings, extended and further precised, especially by JASPER *et al.*, suggested an interpretation for the mechanism of the electroencephalographic spindles usually recorded for instance during natural or barbiturate sleep. Indeed, a striking similarity exists between those spontaneous rhythms and the bursts of waves induced by a slowly repetitive stimulation of the diffusely projecting thalamic nuclei.

On the other hand, it was shown that this non-specific thalamic system is itself controlled by the mesencephalic and bulbar reticular formation, which, when activated, is able to counteract the cortical recruitment. Thus, as a clear ascending influence exists between the rostral and caudal parts of the so-called centrencephalic structures, it seemed interesting to test the reverse possibility, namely a feed-back action of the thalamus upon the brain stem reticular formation. This was done by seeking for recruiting responses in the mesencephalic region.

Cats were prepared under ether anesthesia and paralysed by Flaxedil. Although barbiturates in small doses are known to facilitate the production of recruitment, it seemed advantageous, at least for the preliminary experiments, to eliminate this additional variable. Electrical stimuli (repetitive 1 ms square pulses) were applied by bipolar electrodes inserted in a steel needle. Bipolar electrodes of the same type or glass microelectrodes (about 5 μ at the tip) were used to record the potentials in the central core of the mesencephalon at the Horsley-Clarke frontal plane 2 and lateral planes 1 to 3.

Various types of responses were elicited in the mesencephalic region upon repetitive stimulation of different subcortical and cortical structures. Among them, the typical patterns described here as 'recruiting responses' fulfilled the following criteria, adopted by JASPER and AJMONE-MARSAN for similar phenomena recorded on the cerebral cortex³:

(1) While the first of a series of stimuli at 8 or 10/s produced little or no visible potential, a maximum amplitude of the responses was reached after the third to the sixth repetitive shock; then the amplitude gradually decreased to a more or less constant value.

(2) The optimum frequency of stimulation was found to lie between 5 and 12.5/s. At lower frequency, the potential waves escaped the driving by the afferent impulses. At higher frequency, recruitment simply did not occur.

(3) The latency of the responses was about 20 to 50 ms.

The records of Figure A and B illustrate the given definition. Such patterns may be easily obtained from stimulating points anywhere in the diffusely projecting nuclei of the thalamus. Moreover, when cortical and mesencephalic recordings were simultaneously made, recruitment either appeared at both sites or did not appear at all, according to the location of the stimulating elec-

trodes. On many occasions, a shock intensity of 1.5 V was sufficient for the production of good responses.

Other observations were also made, which can be summarized as follows:

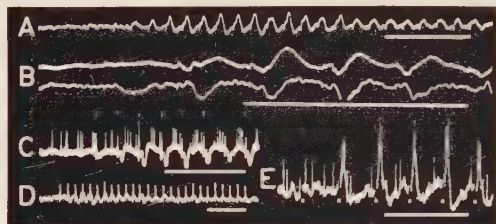
(1) Reticular units, responding to various and widespread peripheral stimulations, most often also discharged in relation with the local recruiting waves. The characteristics of their induced firing have not yet been fully analysed, but it appears that several patterns of response may be distinguished on the basis of both latency and relation with the polarity of the slow potentials. Two typical examples are presented in Figure C and E.

(2) Under favourable conditions, it was sometimes possible to elicit mesencephalic recruiting responses by stimulation of the cerebral cortex.

(3) However, recruiting responses were still elicitable from the thalamus into the mesencephalon after removal of the whole cerebral cortex of both hemispheres (Fig. D).

(4) Effective sites of stimulation were found exclusively in and around the area of the non-specific thalamic nuclei.

(5) Although no systematic exploration of the receptive field in the brain stem has been so far achieved, it may be stated that the recruiting responses, when occurring, seemed to extend broadly in the whole field of the medial mesencephalon.



Oscillographic records of recruiting responses in the mesencephalic reticular formation. A Recording point inferior and lateral to griseum centrale, 10/s stimulation of nucleus centralis lateralis with intensity 1.5 V. – B Upper trace from prefrontal cortex, lower trace from medioventral mesencephalon, parallel development of recruiting waves at both sites by stimulating at 6/s at the border between nucleus ventralis lateralis and nucleus paracentralis. C Mesencephalic reticular unit discharging in relation to the recruiting waves, 8/s stimulation of nucleus centralis lateralis. D Mesencephalic recruitment upon 10/s stimulation of nucleus centralis medialis after total decortication of both hemispheres. E Short latency unitary responses in the mesencephalic reticular formation upon 8/s stimulation of nucleus paracentralis. All time marks: 0.5 s except for trace E where it is 0.3 s.

(6) A high frequency stimulation (100/s and more) of the cerebral cortex and single or repetitive shocks applied to a peripheral nerve might reduce the amplitude or even suppress the production of recruiting responses.

These experiments demonstrate the back action of the diffusely projecting system of the thalamus on mesencephalic regions which are physiologically considered as belonging to the reticular system. Faced with this fact, one would naturally inquire about the significance and importance of this feed-back mechanism. The problem has been approached by studying the interaction between impulses of central and peripheral origin, elicited at various time intervals by stimulation of the thalamus and cutaneous nerves. The first attempts indicated that these two types of afferents do not activate the reticular neurones in the same way. So the resulting figure of interaction cannot be easily interpreted in simple terms of occlusion and facilitation. Further complication is brought

¹ This work has been sponsored by the Office of Air Research and Development Command, U.S. Air Force, under contract No. A.F. 61(514)-22.

² E. W. DEMPSEY and R. S. MORISON, *Amer. J. Physiol.* 135, 293 (1942).

³ H. H. JASPER and C. AJMONE-MARSAN, in *Patterns of organisation in the central nervous system* (Williams and Wilkins Co., Baltimore 1952), p. 493.

in by the reciprocal action of central and peripheral stimuli because of the mutual alterations induced by signals in these two channels. Nonetheless, the present interest in this field¹ is expected soon to clarify our understanding of the descending influences on the ascending activating reticular system.

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Résumé

La stimulation des noyaux non-spécifiques du thalamus chez le chat non anesthésié, provoque des réponses de recrutement dans le mésencéphale. Il est démontré que ces réponses sont analogues à celles qu'on enregistre classiquement sur le cortex et que les neurones réticulés participent à cette activité induite. Ces résultats impliquent l'existence de relations réciproques entre les structures diencéphaliques et mésencéphaliques qui contrôlent le niveau d'activation cérébrale.

¹ W. R. ADEY, J. P. SEGUNDO, and R. B. LIVINGSTON, *J. Neurophysiol.* 20, 1 (1957).

Inhibition of Anaphylactic Shock by Oral Lipids

In the course of a comparative study on the mechanism of action of anti-allergic substances undertaken in our laboratories, it was found that dietary lipids can produce an immediate anti-allergic effect inasmuch as guinea-pigs may be protected against lethal anaphylactic shock within 1 h after the ingestion of certain lipids. The following is a preliminary account of these experiments.

Methods.—Male guinea-pigs weighing from 300–350 g were sensitized by a single subcutaneous dose of 0.5 ml of a 20% (v/v) egg-white solution. Three weeks later, anaphylactic shock was elicited by an intravenous injection of 0.2–0.3 ml of a 2% (v/v) egg-white solution. This procedure resulted in the death of 39 out of a total of 47 control animals and never killed less than 3/4 of the actual control group run in the same series. The egg-white used in these experiments was obtained from several fresh eggs; it was homogenized by repeated freezing and thawing and straining through gauze. Identical batches were used for sensitization and challenging. The lipids to be tested were warmed to 38–40°C and fed drop-wise with a pipette. In most cases, the amount administered was 2 ml/animal and the interval between feeding and challenging 60 min. In a few cases the interval between feeding and challenging or the amount of lipid fed were varied. Oils and fats were employed as available from various commercial sources. Cod-liver and sesame oil were of the type specified by the Swiss Pharmacopoea (Ph. H. V.). Linoleic and linolenic acid (Hoffmann-La Roche) were obtained from ampoules sealed under nitrogen and mixed with the specified amounts of arachis oil or propylene glycol *ex tempore* 5 min prior to being fed.

Results (see Table). — A single oral dose of certain lipids exerted a protective effect against lethal anaphylactic shock in the guinea-pig. The list of active lipids comprises three oils of vegetable origin, i.e. cotton-seed, sesame and corn oil, and one animal fat, i.e. bone oil. The effects obtained with arachis oil, linolenic acid and cod-liver oil are probably not significant in view of the restricted number of animals employed. Olive oil, soy bean lecithin mixed with arachis oil, linoleic acid, fresh cream, butter, lard and egg-yolk were ineffective in the amounts studied.

From the experiments performed with corn oil it is evident that a minimum of lipid must be fed in order to obtain protection. With the material employed here, the minimal dose would seem to lie between 1 and 2 ml.

The lacteals of animals dying from anaphylactic shock within 5 to 20 min after challenge, i.e. 65 to 80 min after feeding of the lipid were visible as white turgescient strings; in a few cases, the serum taken from such animals at death was slightly opaque. Likewise, in cases where 2 ml amounts of corn oil etc. were fed to non-sensitized guinea-pigs or to guinea-pigs sensitized but not challenged; the lacteals showed the same whitish content 90 min after receiving the lipid. Of the sensitized guinea-pigs protected against lethal anaphylactic shock by corn oil or by sesame oil, a few were killed in order to examine the lacteals after the animals had recovered, i.e. 20–30 min after challenging. In all these protected animals, the lacteals were entirely transparent. It would thus appear that during anaphylactic shock in the guinea-pig some clearing agent is released—most probably from the intestinal tract.

Lipids	Amount fed* (ml)	Protection**
<i>Vegetable</i>		
Arachis oil	2	3/10
Cotton-seed oil	2	6/10
Sesame oil	2	7/14
Olive oil	2	2/10
Corn oil	2	7/13
	1	2/7
	0.5	1/5
Soy bean lecithin	1	2/10
plus arachis oil	1	
Linoleic acid	0.5	1/8
plus arachis oil	1.5	
Linolenic acid	0.5	2/5
plus propylene glycol . . .	1.5	
<i>Animal</i>		
Fresh cream	2	2/8
Butter	2	1/9
Lard	2	2/8
Egg-yolk	2	2/9
Cod-liver oil	2	3/8
Bone oil	2	4/10
Controls (no feeding)	—	8/47
Controls: Peptone 10% . . .	2	1/9

* Oral administration of lipids etc. 1 h before challenging.
** Number of animals protected/number of animals employed.

From further experiments with corn oil in 2 ml amounts it became evident that the protective activity of this lipid sets in when absorption has reached a certain level, i.e. in this case not until 30–45 min have passed; it then lasts for about 2 to 3 h and is practically finished 5 h after feeding the lipid.

Preliminary but unequivocal experiments in which groups of sensitized guinea-pigs were fed with corn oil and simultaneously injected with anti-anaphylactic doses of *Proteus* polysaccharides¹ or antihistamines, such as tripeleennamine or promazine, showed that feeding of the anti-anaphylactically effective lipid together with an injection of the anti-allergic polysaccharide resulted in no

¹ R. MEIER, H. J. BEIN, and R. JAKES, *Exper.* 12, 235 (1956); *Int. Arch. Allergy* 11, 101 (1957).

protection at all, i.e. in a mortality identical with that for non-treated controls. On the other hand, all the animals receiving the lipid by mouth and an antihistamine parenterally were fully protected.

The nature of the protective materials present in the lipids studied and the mechanism by which they exert their effect are as yet unknown. Since such lipids as egg-yolk, soy bean lecithin, or linoleic acid were found to be ineffective against anaphylactic shock, the type of lipid activity observed is not directly dependent on phospholipids or unsaturated fatty acids as such, nor is it related to the arachis oil factor described by D. A. LONG and MARTIN² or to the egg-yolk fractions active in the dietary experiments of COBURN *et al.*³ or CHANG and FRENCH⁴. The anti-anaphylactic activity of sesame, cotton-seed oil etc. is furthermore clearly distinguished by the fact that one single oral dose is effective whereas in the experiments of the last-named authors the lipid had to be injected or fed over several weeks in order to suppress tuberculin or Arthus reactions in the guinea-pig.

It is a pleasure to acknowledge the valuable technical assistance given by Miss E. HEITZ.

R. JAQUES

Research Laboratories of the Pharmaceutical Department of CIBA Limited, Basle (Switzerland), May 27, 1958.

Zusammenfassung

Gewisse Lipide besitzen am Meerschweinchen nach einmaliger peroraler Verabreichung eine mehrstündige anti-anaphylaktische Schutzwirkung. Der anti-anaphylaktische Effekt von parenteral zugeführten Proteus-Polysacchariden wird durch perorale Zufuhr solcher Lipide aufgehoben, während Antihistaminica in ihrer Schutzwirkung nicht beeinflusst werden.

² D. A. LONG and A. J. P. MARTIN, *Lancet* 270, 464 (1956).
³ A. F. COBURN, C. E. GRAHAM, and J. HANINGER, *J. exper. Med.* 100, 425 (1954).
⁴ I. CHANG and C. E. FRENCH, *Proc. Soc. exp. Biol. Med. N.Y.* 93, 366 (1956).

Organ Weights of TE Mice Bearing Ehrlich Ascites Carcinoma Growing in the Solid Form and of C3H Mice Bearing Spontaneous Mammary Carcinoma

In the course of our studies on the anaemia of cancer, we observed that the weight of the spleen and the liver of cancerous mice was higher than the weight of the spleen and the liver of healthy mice.

MÜLLER¹ reported that in humans who died from various carcinomas the weight of the spleen sometimes though not always was significantly higher than the weight of the spleen of non-cancerous individuals (cf. also CALO²).

In this communication we report observations on the weight of spleen, liver and kidneys of TE mice bearing Ehrlich ascites carcinoma in the solid growing form and of C3H mice bearing spontaneous mammary carcinoma.

Male TE mice, kindly supplied by the Animal Department of the British Medical Council, were inoculated intramuscularly with Ehrlich ascites carcinoma and killed as described elsewhere³. The spleen and liver were removed and weighed. In one series the dry weight of the organs was also determined.

¹ IRMA MÜLLER, *Zbl. allg. Path. path. Anat.* 55, 180 (1932).
² A. CALO, *Z. Krebsforsch.* 37, 151 (1932).
³ G. V. EHRENSTEIN, *Arkiv Kemi* (1958), in press.

Table I
Fresh weight of spleen, liver and kidneys of female C3H mice bearing spontaneous mammary carcinoma and of healthy male C3H mice.

	Number of animals	Mean weight in g	Standard error	Range
<i>Controls</i>				
Body weight. .	11	28	± 1.7	21–36
Spleen weight. .	11	0.183	± 0.016	0.125–0.269
Liver weight. .	11	1.654	± 0.086	1.243–2.026
Kidney weight. .	11	0.548	± 0.047	0.332–0.791
<i>Tumours*</i>				
Body weight. .	11	30	± 1.3	23–37
Spleen weight. .	11	0.540	± 0.041	0.271–0.726
Liver weight. .	11	2.178	± 0.139	1.631–2.871
Kidney weight. .	11	0.386	± 0.011	0.340–0.461

* Average tumour weight = 2.5 g.

The C3H mice were females of about 6 months of age all having spontaneous mammary carcinoma. In order to get controls of this strain we were obliged to take C3H males of the same age and body weight. In this series the weight of the kidneys was also determined.

The results are seen in Table I and II. The fresh weight of the spleen in both tumour groups is significantly higher than in the controls, the ratio between cancerous and control mice being 3.7 for the TE mice and 3.0 for the C3H mice. The enlargement of the spleen was proportional to the tumour weight. The corresponding figures for the liver are 1.4 and 1.3. For the dry weights essentially identical ratios were obtained. The weight of the kidneys of the cancerous C3H mice was found to be lower than that of the controls, the ratio being 0.7. When the C3H females were allowed to die spontaneously, the dispersion of the values was greatly increased, some animals were found to have a normal, some even a decreased liver and spleen weight. For these results cachexia was presumably responsible.

Tissue examination revealed no metastases. Histological examination showed the presence of massive infiltrations of plasma cells or lymphocytes in the spleen and periportal infiltrations of plasma cells or lymphocytes in the liver of the cancerous mice (cf. CALO²). Our radioiron studies suggest that there must also be an appreciable amount of erythropoietic tissue in the spleen of the cancerous animals (v. EHRENSTEIN³, see also KELSALL⁴ and ANTOPOL *et al.*⁵).

We have also injected healthy male TE mice intraperitoneally under aseptic conditions with 0.25 ml of whole blood of TE mice bearing Ehrlich ascites carcinoma in the solid growing form. The weight of the spleen of the healthy mice, which received the blood of the cancerous animals, was found to be increased to twice that of the controls which were injected intraperitoneally with saline. The weight of the liver and kidneys was not affected in these experiments. Intraperitoneal injection of 0.25 ml of whole blood of healthy mice did not produce splenomegaly.

Intraperitoneal injection of 0.2 ml of blood plasma of cancerous TE mice into healthy TE mice caused spleen enlargement to twice the normal weight as early as 120 h after the injection. Intraperitoneal injection of either 0.2 ml plasma or 0.1 ml packed red blood corpuscles of healthy TE mice, or of 0.1 ml of packed red corpuscles of cancerous TE mice, did not affect the spleen weight.

⁴ M. A. KELSALL, *J. nat. Cancer Inst.* 10, 625 (1949).
⁵ W. ANTOPOL, S. GLAUBACH, and S. GRAFF, *Proc. Amer. Assoc. Cancer Res.* 2, 276 (1958).

Table II
Weight of spleen and liver of male TE mice bearing Ehrlich Ascites Carcinoma growing in the solid form and of healthy male TE mice.

	Number of animals	Mean weight in g	Standard error	Range
<i>Controls</i>				
Body weight.	36	32	± 0.5	26-36
Spleen {	fresh weight	36	± 0.007	0.066-0.208
	dry weight	10	± 0.001	0.018-0.031
Liver {	fresh weight	36	± 0.030	1.053-2.258
	dry weight	10	± 0.021	0.334-0.538
<i>Tumours*</i>				
Body weight	33	35	± 0.5	27-40
Spleen {	fresh weight	33	± 0.027	0.124-0.706
	dry weight	11	± 0.007	0.033-0.100
Liver {	fresh weight	33	± 0.054	1.629-2.775
	dry weight	11	± 0.020	0.426-0.662

* Average tumour weight = 2.5 g.

These results compare with the findings of CROSSLEY *et al.*⁶ in rats.

It is improbable that the enlargement of the spleen caused by intraperitoneal injection of plasma from cancerous mice was produced by an infection in the cancerous animals (cf. ANTOPOL *et al.*⁵). Studies are in progress in this laboratory on this problem.

The author's thanks are due to Professor G. HEVESY for stimulating interest taken in this work, to Professor G. KLEIN for kindly supplying the Ehrlich ascites carcinoma and the C3H mice, to Professor G. HÄGGQVIST for preparing and examining the histological preparations, to the Riksföreningen för kräftsjukdomarnas bekämpande, Cancernämnden, and the Knut och Alice Wallenbergs Stiftelse for their generous support of this investigation.

G. V. EHRENSTEIN

Institute of Organic Chemistry and Biochemistry, University of Stockholm, May 5, 1958.

Zusammenfassung

Das Milzgewicht karzinomtragender Mäuse ist dreimal so hoch wie das Milzgewicht gesunder Kontrollen.

Die Leber karzinomtragender Mäuse ist ungefähr anderthalbmal so schwer wie die Leber gesunder Kontrollen.

Das Nierengewicht von C3H-Mäusen mit Mammakarzinom beträgt nur etwas mehr als die Hälfte des Nierengewichtes gesunder Kontrollen.

Intraperitoneale Injektion von 0,25 ml Vollblut oder 0,2 ml Plasma von TE-Mäusen mit Ehrlich-Ascites-Karzinom in der solid wachsenden Form erhöht das Milzgewicht gesunder Kontrollen auf das Doppelte.

⁶ M. L. CROSSLEY, M. BRUSH, F. JENCKS, and J. B. ALLISON, Proc. Amer. Assoc. Cancer Res. 2, 11 (1955).

Über die Reaktion der höheren und tieferen Reizbildungszentren des isolierten Herzens auf Temperatursenkung

In der Literatur liegen zahlreiche Angaben vor über einen engen Zusammenhang zwischen Herzfrequenz und Körpertemperatur. Kürzlich wurde von BADEER¹ sogar

eine lineare Beziehung zwischen diesen beiden Faktoren im Intervall von 25 bis 40°C beschrieben. Dagegen sind unsere Kenntnisse über die Reaktion der tieferen Reizbildungszentren bei Hypothermie sowie über die gegenseitige Beziehung der temperaturbedingten Aktivitätsänderungen der einzelnen Reizbildungszentren sehr gering.

Zur Klärung dieser Fragen wurde an nach LANGENDORFF isolierten Kaninchenherzen das Hissche Bündel durchtrennt und die Änderungen in der Tätigkeit der Vorhöfe bzw. der Kammern elektrokardiographisch registriert. Die Änderung der Vorhoffrequenz war für die Aktivität des Sinusknotens, die Änderung der Kammerfrequenz für die tertiäre Kammerreizbildung kennzeichnend. Änderungen der atrioventrikulären Reizbildung konnten nach Elektrokoagulation oder nach mechanischer Schädigung des Sinusknotens untersucht werden; die Führung wird nach einem solchen Eingriff durch das atrioventrikuläre Reizbildungszentrum übernommen; die Eigenfrequenz der Vorhöfe kann als Index der atrioventrikulären Reizbildung aufgefasst werden. Einzelheiten der Methodik sind in einer anderen Mitteilung (SZEKERES, FALLER und LICHNER²) ausführlich beschrieben worden.

Bei dieser Versuchsanordnung nahm die Aktivität sowohl der homotopen wie auch der heterotopen Reizbildung bei Kühlung der Durchströmungsflüssigkeit deutlich ab. Bei 26°C sank die Kammerfrequenz durchschnittlich auf 40%, die atrioventrikuläre Frequenz auf 31% und die Sinusfrequenz auf 28,3% des bei 38°C beobachteten Ausgangswertes. Die Abbildung veranschaulicht einen solchen Versuch. In der Tabelle sind die Versuche über die Reaktion der einzelnen Reizbildungszentren bei Durchströmung mit Lockelösung von 38°C bzw. 26°C zusammengefasst. Wie ersichtlich, reagiert der Vorhof (Sinusreizbildung) mit der stärksten Frequenzverminderung, die Frequenzabnahme der atrioventrikulären Reizbildung ist bedeutend kleiner und diejenige der Kammern am geringsten. Die Unterschiede in der temperaturbedingten Frequenzabnahme einzelner Reizbildungszentren sind statistisch gut gesichert. Diese Versuchsergebnisse weisen darauf hin, dass die Sinusreizbildung auf Senkung der Temperatur empfindlicher reagiert als die «tertiäre» Kammerreizbildung. Die atrioventrikuläre Reizbildung steht zwischen diesen beiden.

² L. SZEKERES, J. FALLER und G. LICHNER, Arch. exp. Path. Pharmacol. 233, 343 (1958).

¹ H. BADEER, Amer. J. Physiol. 167, 76 (1951).

Reaktion der höheren und tieferen Reizbildungszentren des isolierten Herzens bei Durchströmung mit Lockelösung von 38°C bzw. 26°C

	n	Frequenz/min			t	p
		38°C	26°C	Frequenzabnahme		
Vorhof	20	151,35 ± 8,65	42,90 ± 4,00	108,45 ± 7,10}	4,52	< 0,001
Atrioventrikularknoten	9	83,10 ± 6,11	25,80 ± 2,03	57,30 ± 5,61}	7,14	< 0,001
Kammer	20	48,35 ± 4,01	19,35 ± 2,23	29,00 ± 2,49}		

Ein Beispiel für die Änderungen der Vorhoffrequenz, der atrioventrikulären Frequenz und der Kammerfrequenz bei Abkühlung der Durchströmungsflüssigkeit.

Es soll hier noch erwähnt werden, dass an Langendorff-Heizen mit durchschnittlichem Hisschem Bündeln in einigen Fällen bei tiefer Temperatur die Vorhöfe sich langsamer kontrahierten als die Kammern. Manchmal konnte eine regelmässige Kammertätigkeit auch bei völligem Vorhofstillstand beobachtet werden. Durch diese höhere Empfindlichkeit der Sinusreizbildung ist die Möglichkeit einer Erklärung für die von GROSSE-BROCKHOFF und SCHOEDEL³ am EKG des Hundeherzens *in situ* beobachtete Erscheinung gegeben, dass nämlich bei Senkung der Körpertemperatur unter 25°C die Führung der Herztätigkeit durch die atrioventrikuläre Reizbildung übernommen wird.

In unseren früheren Untersuchungen (SZEKERES, FALLER und LICHNER²) haben wir gezeigt, dass in Hypoxie wie auch in Hyperkapnie die «tertiäre» Kammerreizbildung am empfindlichsten reagiert, die atrioventrikuläre Reizbildung schwächer und die Sinusreizbildung am wenigsten beeinflusst wird. Hier liegen somit gerade umgekehrte Verhältnisse vor wie in den oben beschriebenen Versuchen. Es ist folglich nicht wahrscheinlich, dass die bei Temperatursenkung beobachteten Empfindlichkeitsunterschiede der einzelnen Reizbildungszentren auf einen etwaigen Sauerstoffmangel oder Kohlesäureüberschuss zurückzuführen sind.

L. SZEKERES und G. LÉNÁRD

Pharmakologisches Institut der Medizinischen Universität Pécs (Ungarn), 28. April 1958.

Summary

In the isolated rabbit heart perfused by the Langendorff method total heart block has been produced by section of the His bundle. The influence of cooling of the perfusion fluid upon higher and lower automatic centers was studied. The sinoauricular pacemaker responded to cooling with a much greater decrease of the heart rate than did the ventricular automatism. The responsiveness of the atrioventricular node to cooling lay between that of the two others.

³ F. GROSSE-BROCKHOFF und W. SCHOEDEL, Arch. exp. Path. Pharmac. 201, 417 (1942).

PRO LABORATORIO

Anxiety and Learning in Rats under Stress-Avoidance-Discrimination Conditions as Influenced by Chlorpromazine, Reserpine, and Glutamate

The methods of measurements of the central activity of the ataraxics have been most diverse. A clear differentiation from other central depressants is not always possible. One popular means of measuring responses to tranquilizers is a shock-avoidance apparatus in which animals learn to react to a warning auditory or visual stimuli such as a buzzer or light. Failure to heed this warning results in a punishment by an electric shock. Animals learn to escape when this warning stimulus is applied. The fear and/or anxiety which is initiated by this warning stimulus may be abolished by the administration of various central depressants. An animal thus treated will not escape until it has received the shocking stimulus.

We wished to add the factor of discrimination to these responses. A shock-avoidance box was designed in such a manner so that discrimination is necessary for avoidance of shock (Figure). In this apparatus there is a main compartment the floor of which consisted of metal bars. By this design a shock (5 mA) was administered to the test animal 15 s after a warning light was turned on. At the same time as the compartment was lighted a guillotine type of door was raised, which offered to the animal two possible exits. One of the exit-compartments contained a low-voltage light and the other was darkened. The lighted escape compartment was unsafe as the floor of this 'room' was also covered with metal bars which continued the shock for 15 s if the rat chose to jump into it. The other compartment was a safe exit of escape since the floor was covered with a removable smooth insulating material. The position of the safe compartment was changed in random order every one to six trials. The total number of right and left escape routes were equal. If a rat failed to leave the main compartment during the 15 s in which the light was on, a shock of 5 mA was administered for 15 s. If this shocking stimulus was not effective the door was lowered and another 15 s of shock was administered. In this experiment the total number of trials per animal was 56, over a period of 29 days.

With this plan we have measured and recorded the following data: (1) The number of trials in which the animals did not respond to the stimulus or the light i.e. they took the shock (TS), (2) The responses to light that were correct (L+) and those that were incorrect (L-), (3) S+ or the total correct responses to the shocking stimulus and the incorrect responses, S-. Correctness means the escape to the safe exit compartment. The total responses to the light would therefore be L+ plus L-. L+ plus S+ will then give us a total measurement of the discriminatory reaction of the test animal.

Weanling untrained male rats were used. Reserpine, chlorpromazine, and monosodium glutamate were administered orally every day. The tranquilizers chosen have unrelated chemical structure and presumably different



sites of action. Many of the pharmacological effects of these drugs are also different. In a previous report we have found that glutamate increased the potentiating property of reserpine but not of the phenothiazine type of drug¹. Each of the drugs was diluted with distilled water and given by mouth *per os* 1 h before testing. A 3 × 3 factorial design was used.

- The factors were:
- (a) ataraxics, 3 levels, 0,2 mg/kg reserpine, and 2 mg/kg chlorpromazine;
 - (b) monosodium glutamate, 3 levels, 0,1 g/kg, and 2 g/kg.
- Nine rats were used per block, a total of 81 rats.

Results. Table I represents the six analyses of a variance that were made. There was no difference in the initial weight of the group.

Table II represents the performance of each group in percentage form. Reserpine depressed the number of responses to light, both discriminatorily correct (L+) and

¹ E. KOPMANN and F. W. HUGHES, *Proc. Soc. exp. Biol. Med.* 97, 83 (1958).

Table I

P-values of Analysis of Variance for:				
Source-F-Ratios	L +	TS	L + plus S +	L + plus L -
MSG-1 g/kg .	n.s.	n.s.	n.s.	n.s.
MSG- 2 g/kg .	n.s.	n.s.	n.s.	n.s.
Reserpine				
2 mg/kg . . .	decrease < 0.02	increase < 0.05	decrease < 0.05	decrease < 0.05
Thorazine				
2 mg/kg . . .	n.s.	n.s.	n.s.	n.s.

Reserpine decreased the number of total correct responses made to light and it increased the total number of times that the animals took shock (TS). These animals were all conscious and physically able to respond.

incorrect (L -). Since the time interval that the light is on preceeds the shock, it can be considered as a response to an indirect, or derived stress and has been considered to be a measure of anxiety. Thorazine and glutamate did not effect this. Reserpine also depressed the total number of correct responses both from indirect stress or light (L+) and from direct stress or shock (S+). This correctness in escape route *in toto* (L+ plus S+) we consider as a measure of discrimination.

Experiments are in progress in which we will further measure changes in this discriminatory capacity of animals under the influence of various tranquilizers and other depressants. The extinction of this learned-discrimination response by various compounds may aid in the isolation of distinct activity of these ataraxics.

An interesting observation was that the mean weight gain in the groups treated with chlorpromazine was greater (106 g) than the control group (101 g). This was not of a significant nature in this series of animals. However the animals receiving reserpine showed a lesser weight gain (74 g) than the control group (101 g). This was highly significant (P < 0.001).

E. KOPMANN and F. W. HUGHES

Department of Pharmacology, Indiana University School of Medicine, Indianapolis, May 23, 1958.

Zusammenfassung

Ein neuer Apparat wird beschrieben, in welchem die Wirkungen von Chlorpromazin, Reserpin und Natriumglutamat auf Furcht und Lernen in Ratten untersucht wurden.

Table II. Total responses of rats on % basis. Drugs administered daily via oral route

Treatment	Dosage	TS %	S - %	L - %	S + %	L + %
Water	2 cm ³	14	4	27	8	47
Monosodium Glutamate (MSG)	1 g/kg	20	9	21	9	41
MSG	2 g/kg	21	10	19	8	42
Reserpine	2 mg/kg	25	9	23	9	34
Reserpine and MSG	2 mg/kg	38	7	21	8	26
	1 g/kg					
Reserpine and MSG	2 mg/kg	28	7	23	7	35
	2 g/kg					
Chlorpromazine	2 mg/kg	21	7	23	7	42
Chlorpromazine and MSG	2 mg/kg	9	11	28	8	44
	1 g/kg					
Chlorpromazine and MSG	2 mg/kg	23	8	23	13	33
	2 g/kg					

TS = Take shock; No attempt to escape upon shocking. S+ = Correct response to shock. S- = Incorrect response to shock.
L+ = Correct response to light. L- = Incorrect response to light.

Informations - Informationen - Informazioni - Notes

STUDIORUM PROGRESSUS

Production *in vitro* of Melanin-Like Pigmentation by Cells of the Intestinal Mucosa¹

By H. LANGEMANN² and G. B. KOELLE³

The dendritic melanocytes of the epidermis and the cells of the hair matrix contain melanin granules, the amount and/or size of which is increased by the classical dopaoxidase reaction as described by BLOCH and RYHNER⁴ and BLOCH⁵, or by the tyrosinase reaction (FITZPATRICK and coworkers⁶). In the full series of reactions, beginning with the oxidation of tyrosine and leading to the appearance of cytoplasmic melanin granules, visible with the light microscope, five steps can be considered separately in accordance with current proposals.

The oxidation of tyrosine to dopa, and secondly from dopa to dopachrome is possible through autoxidation of tyrosine, and can be accelerated considerably by small amounts of dopa (LERNER⁷). It is catalyzed by phenol-oxidase from mushroom extracts, which is a copper-containing enzyme. Recently the two steps were separated, the first being catalyzed by cresolase, the second by catecholase, both of which are copper-containing proteins (MASON⁸). These two steps require 1 atom of oxygen each. In spite of numerous investigations, the third step (further oxidation of dopachrome to melanin), the structure of melanin, and the fourth step (i.e. the polymerization of melanin) are not completely understood. According to MASON⁹ 2.6 more atoms of oxygen are required. MONDER, WILLIAMS, and WAISMAN¹⁰ found that 6 atoms are required. Both groups state that 1 molecule of CO₂ is given off in the course of the oxidation. However, according to CROMARTIE and HARLEY-MASON¹¹ dopa-melanin still contains about 1/8 of its original CO₂. The fifth step, the formation of melanin protein, resulting in microscopically visible granules in the cytoplasm of melanocytes, is also poorly understood. There is no certainty as to whether these five steps are actually catalyzed enzymatically in this exact sequence in animal tissues, or if other reactions lead to their completion.

In the course of experiments on the histochemical detection of aromatic amines, the development of pigmentation in certain cells in the lamina propria of the mucosa of the small intestine of guinea pigs was observed when sections were incubated for short periods in buffered solutions containing 5-hydroxytryptophane. Incubation in

5-hydroxytryptamine solutions resulted in a more pronounced pigmentation. A number of amines and amino acids of the indolalkylamine or the catecholalkylamine series was then investigated. With 3-hydroxytyramine (dopamine) and with dihydroxyphenylalanine (dopa) a very striking reaction was observed. It was then decided to investigate whether this phenomenon was related to the dopaoxidase. It was also hoped to learn more about the phenolic oxidation and the possible role in melanin-formation of these physiologically and pharmacologically active compounds. A number of questions arose in the course of this investigation which cast some doubt on the completeness and the validity of the currently existing theories of the dopaoxidase or tyrosinase reactions as described for skin, which may require reevaluation of the proposed individual steps of these reactions. For this reason it seemed justified to publish these preliminary results at any early stage. Much more must be done to clarify the situation.

Methods.—Most studies were performed with small intestine, generally duodenum and less frequently ileum or jejunum. Other parts of the gastrointestinal tract investigated include stomach, cecum, appendix, ascending and descending colon. The other organs or tissues examined were skin, kidney, liver, brain, heart, spleen, adrenal gland, thyroid gland, and thymus.

Tissues of adult guinea pigs of either sex were used in the majority of the experiments; the remainder were taken from rats, and but a few from man and frog. The animals were sacrificed by stunning and bleeding. Human tissues were obtained shortly after surgery (appendix, normal and inflamed; through the courtesy of Drs. H. T. ENTERLINE and J. J. MORAN, Department of Pathology, Hospital of the University of Pennsylvania).

Frozen sections of fresh or formalin-fixed tissues (10% buffered formalin) were cut usually at 10 μ , or occasionally at 20 or 30 μ , and placed directly on slides. The incubation medium consisted of 10 ml of 0.1 M phosphate buffer containing 0.72% NaCl, or of 0.0067 M phosphate buffer (Soerensen), pH = 8.0, plus substrate. Tissues were incubated in covered Coplin jars at 37°C, or at room temperature (25–29°C). The incubation time was usually 30 min, except when noted otherwise.

The following substrates were used, mostly at a concentration of 0.002 M, occasionally at 0.005 M or 0.01 M: dopamine HCl, L-dopa or DL-dopa, epinephrine bitartrate, levarterenol bitartrate monohydrate, tyramine HCl, DL-tyrosine (not dissolved completely), 5-hydroxytryptamine creatinine sulfate, DL-5-hydroxytryptophane, tryptamine HCl, L-tryptophane. In certain experiments the solutions were bubbled through with N₂ (freed from O₂ by bubbling through pyrogallol solution).

Immediately after the incubation period the sections on the slides were covered with glycerine; alternatively, some were passed through alcohols, xylol and preserved in 'Permunt' after counterstaining with hematoxylin-eosin.

Results.—A number of changes in the experimental histochemical conditions were made in the attempts to classify the pigmented cells, and to clarify the underlying biochemical mechanism. Under the experimental conditions described above, the following typical result was obtained consistently when sections of duodenum were incubated at a pH of 8 and room temperature for 30 min in a solution of dopamine that had been allowed to autoxidize for a short period of time (see Figure).

¹ This investigation was supported by research grants [B-282 (C3 and C4)] from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service.

² Department of Pharmacology, University of Zürich.

³ Department of Physiology and Pharmacology, Graduate School of Medicine, University of Pennsylvania.

⁴ B. BLOCH and P. RYHNER, *Z. exp. Med.* **5**, 177 (1917).

⁵ B. BLOCH, *Handbuch für Haut- und Geschlechtskrankheiten*, vol. 1, part. 1 (Springer, Berlin 1927).

⁶ T. B. FITZPATRICK, S. W. BECKER, A. B. LERNER, and H. MONTGOMERY, *Science* **112**, 223 (1950).

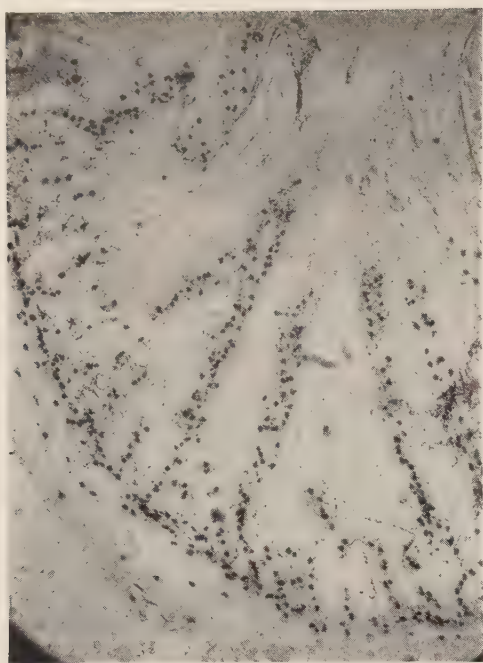
⁷ A. B. LERNER, *Amer. J. Med.* **19**, 902 (1955).

⁸ H. S. MASON, *Nature* **177**, 79 (1956).

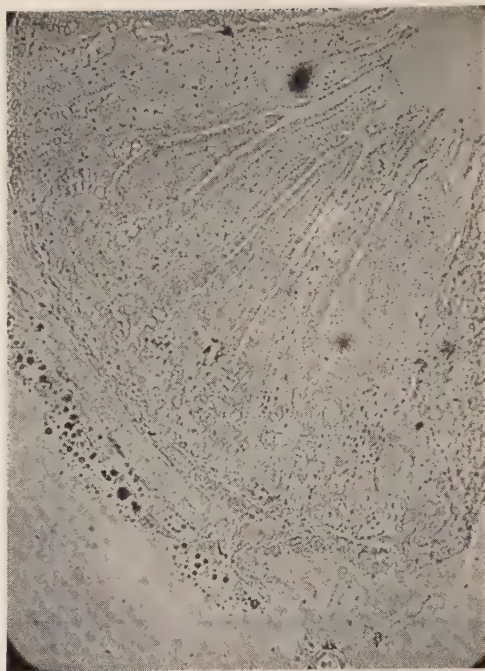
⁹ H. S. MASON, *Adv. Enzymol.* **16**, 105 (1955).

¹⁰ C. MONDER, J. N. WILLIAMS, and H. A. WAISMAN, *Arch. Biochem. Biophys.* **72**, 255 (1957).

¹¹ R. I. T. CROMARTIE and J. HARLEY-MASON, *Biochem. J.* **66**, 713 (1957).



Dopamine incubation



Control

Duodenum of rat, fresh frozen 10 micron sections, dopamine $5 \times 10^{-3} M$, 30 min, pH = 8.7, 22°C. Magnification 74 times
(Photomicrographs by Dr. P. HUBER, Zürich)

The lamina propria of the mucosa, predominantly in the vicinity of the muscularis mucosae and progressively less to the tips of the villi, showed a large number of round or elongated cells of medium size, having an unindented, rather small nucleus, and cytoplasm filled with brown granules of varying size. Some of the cells were homogeneously brown, the nucleus being hidden by the pigment. Some cells were surrounded by a halo of the same pigment. The epithelial parts of the mucosa did not show any of these cells; this was also true for the whole region of the Brunner glands. The muscular layers contained such cells only occasionally. In the sections which had not been incubated or were incubated in a medium devoid of substrate, and thus served as controls, the same apparent kind of cell showed but a very faintly yellow or pale granulated cytoplasmic structure, provided no counterstain was used. They appeared to be eosinophilic.

A similar reaction, and a similar pattern of the distribution of these cells was found in the ileum, jejunum, coecum and to some extent in the pyloric part of the stomach. In the ascending colon only a few such cells were found, and even fewer were noted in the descending colon. In the fundus of the stomach, which contained a few of such cells, some were scattered outside the lamina propria of the mucosa. The appendix showed numerous pigmented cells in the lamina propria mucosae, and also in the other layers, including the subserosa. A huge number of densely packed pigmented cells was found in a highly inflamed appendix. Here the numerous cells in the inflamed regions (connective tissue and muscularis) were larger and showed much more of a pigmented halo than the cells in the lamina propria of the mucosa.

In the following organs some single, scattered, strongly pigmented cells were found but no typical pattern as to their localization could be established: kidney, brain (various parts), heart, and spleen; none was seen in the liver. In a few cases the thymus and thyroid gland showed a large number of pigmented cells. In skin from the ear of brown-red-pigmented guinea pigs or from

albino guinea pigs and rats, the dermis showed a number of strongly pigmented cells. At the same short incubation time (30 min) no increase in granular pigment of the basal epidermal cells or the hair-matrix cells was observed. With an extended time of incubation (overnight), there was some indication of an increase in pigment in the basal epidermal cells as compared to the controls; however accurate quantitation of this was not possible.

In tissues treated with formalin prior to sectioning, leucocytes stained strongly granular, and erythrocytes took up pigment homogeneously though not as much as the leucocytes. This was especially well visible in blood vessels of the inflamed appendix.

Storage of sections on slides in formalin solution for two weeks or for several weeks at room temperature had no apparent influence on the reaction. Subsequent immersion of sections in ethanol or chloroform for 24 h did not affect the pigment formed from dopamine.

The colour which developed in the cells after incubation with dopamine or dopa was brown, dopa yielding a somewhat darker shade. In all cases, a strong pigment reaction was obtained only in the presence of a coloured substrate medium: During the 30 min period of incubation these substrates (at pH 8) turned grey-violet and somewhat turbid, a phenomenon which is also essential in the classical dopaoxidase reaction. After incubation for 13-18 h the solutions turned dark and a marked black precipitate was formed. In these cases, the cells were very strongly, almost black pigmented, the sections were stained overall gray, leucocytes and erythrocytes were stained black.

When other substrates were used, the shade of pigmentation differed: Yellow-brown after adrenaline or noradrenaline, even following prolonged incubation, and light brown, or light red-brown with tyramine, tyrosine, tryptamin, tryptophane, hydroxytryptamine, or hydroxytryptophane. With all these substrates the intensity after short or prolonged incubation at a pH of 8 was much less than in the dopamine or dopa experiments.

After incubation of sections in solutions of dopamine, dopa, or epinephrine which had been oxidized to the corresponding indolequinone by the addition of 4 equivalents of iodine (KOELLE and FRIEDENWALD¹²), the dopachrome from dopamine or dopa resulted in a violet-black stain even after very short incubation, whereas adrenochrome produced a light-yellow pigmentation.

With tryptamine or tyramine, pigmentation was observed to develop even in the absence of visible colour or turbidity of the incubation medium.

Various factors were investigated as to their possible influence on the reaction.

Little or no difference was found for temperatures of 25–29°C (room) or 37°C (incubator). Heating the sections for 30 min at 60°C in 0.9% NaCl solution resulted in little decrease in subsequent pigment-development, whereas heating for 5 min at 85°C abolished the reaction.

Low temperatures, e.g. 4°C, slowed the reaction considerably, with respect to both the development of visible colour in the solution and the appearance of pigmentation in the cells (even after 16 h there was no strong reaction found). However, quite marked pigmentation was observed after 5 min of incubation at 3°C with iodine-oxidized substrates (dopamine, at 0.01 *M* concentration).

Regardless of whether the sections were incubated in freshly prepared dopamine or dopa solutions, or in solutions where autooxidation had been allowed to take place for 30 min, there was a definite latency in the onset of the pigmentation; incubation for 10 min resulted in the development of much less intense pigmentation than the standard 30 min incubation. However, further increase in incubation time beyond 30 min did not seem to produce a proportionate increase in the amount of pigmentation. With lower concentrations than 0.002 *M* (e.g., 10⁻⁴ or 10⁻⁵ *M*) the pigmentation which developed was negligible even after prolonged incubation. No attempt was made to determine the optimal concentration. The pH of the incubation medium was of great importance; at a pH range of 7 to 6, little autooxidation and little pigmentation occurred with dopamine or dopa. However, pigment uptake took place even at pH 5, and very markedly at pH 9, when previously autooxidized solution of dopa had been adjusted to the corresponding pH-figures.

When N₂ was bubbled through the incubation medium in the Coplin jars containing slides, no visible color change in the medium was observed and no pigmentation occurred. When an incubation medium was allowed to autoxidize first, then slides were added and N₂ was bubbled through, no further visible color development was noticed in the solution, but pigmentation developed in the cells which could not be distinguished in intensity from the corresponding reaction under aerobic conditions. This was observed with incubation media containing autoxidized dopamine, dopa, or adrenaline.

In 0.002 *M* solutions of dopa or dopamine, to which 0.004 *M* cysteine HCl or sodium diethyldithiocarbamate had been added before autooxidation had taken place, no visible color change occurred in the solution during the following 30 min, and no pigmentation of the cells was found. When the solutions had been allowed to autoxidize prior to addition of the thiol compounds, then a positive cell-pigmentation reaction was observed. Exposure of sections to cysteine or diethyldithiocarbamate solutions for 30 min prior to incubation in the dopamine medium did not have any noticeable influence on the pigmentation.

Incubation of sections in a suspension of precipitated dopa-melanin, washed by repeated centrifugation, did not

lead to pigmentation of the cells. Likewise, India ink suspensions were not taken up by the cells.

A few exploratory experiments gave evidence for a strongly positive ferric-ferricyanide reaction, according to the technique of SCHMORL (PEARSE¹³), in the cells that had taken up pigment, but not in the controls.

There was evidence also for a strongly positive benzidine-peroxidase reaction, according to the techniques by VILLAMIL and MANCINI (LILLIE¹⁴) and DEROBERTIS and GRASSO (PEARSE¹³), in the pigment-developing cells in untreated and in dopamin-pigmented sections, formalin-fixed or untreated.

Discussion.—The foregoing observations indicate that certain cells, found mainly in the lamina propria of the mucosa of the small intestine, develop intense melanin-like pigmentation after a short period of incubation in partially autoxidized, visibly colored solutions of a number of catechol alkylamines and indol alkylamines or their corresponding amino acids. These cells resemble macrophages or histiocytes, cells belonging to the originally mesodermal reticulo-endothelial system, which are known to be capable, under proper conditions, of phagocytosis or pinocytosis. The phenomenon observed here does not, however, seem to be of the intravitaly occurring type of phagocytosis. India ink particles or washed (and fully oxidized) melanin-particles prepared from dopa were not taken up. Furthermore, the pigmentation of the cells occurred at the same rate in sections subjected to prolonged drying or treatment with formalin. They did not stain with cresylviolet or toluidine blue, and did not take up trypan blue after its injection into the living animal. In comparison with the known types of cells which have been reported to contain melanin-like pigments, the cells under consideration do not resemble particularly the typical melanocytes of epidermis, which are derived from the neural crest, of the pigmented tissues of the eye, or certain nervous tissues. However, they may be related to the cells found in the somewhat vaguely defined clinical state of pseudomelanosis of the appendix (PEARSE¹³) or in melanosis coli of man; or to the tissue-eosinophils.

In the present investigation, no evidence was obtained that any enzymatic process occurred in the tissue sections prior to the formation of colored compounds such as dopachrome or adrenochrome. Controls and additional modifications must be done to rule out the possible dependence on autooxidation for the initial steps in performing the histochemical dopaoxidase experiments with skin as they are described in the literature. It is unlikely that the small amount of tissue per slide incubated in the 10 ml of substrate solution (approximately 0.1 mg of wet weight of tissue, or 0.2 micro mol of nitrogen) contributed to the first two steps of oxidation, from tyrosine, dopa, or dopamine, in the surrounding medium. Survey of the literature on the histochemical reaction for dopa oxidase skin reveals the general assumption of an enzymatic reaction for these first two steps in epidermal cells; on the other hand it has not been ruled out that cells elsewhere than in the epidermis might produce enzymatically such soluble compounds as dopachrome. Since dopa-melanin is insoluble, whereas dopachrome and adrenochrome are readily soluble, the appearance of the former pigment in cells might have resulted from entrance of a soluble precursor or by phagocytosis of an insoluble stage. In the case of 10 micron-frozen sections, practically all of the cells are transected. It has therefore to be kept in mind

¹³ A. G. E. PEARSE, *Histochemistry, Theoretical and Applied* (Little Brown, Boston 1953).

¹⁴ R. D. LILLIE, *Histopathologic Technic and Practical Histochemistry*, 2nd ed. (Blakiston, New York 1954).

¹² G. B. KOELLE and J. S. FRIEDENWALD, *Arch. Biochem. Biophys.* 32, 370 (1951).

that mere adsorption of insoluble compounds could take place, but probably not their active uptake into cytoplasmic granules. Phagocytosis in these experiments seems ruled out by the previous treatment by freezing, followed in some experiments by formalin-fixation or drying.

The fourth and fifth steps, melanin polymerization and binding to proteins, are so little understood that speculation about these reactions in the cells may be permitted. The fact that the pigment-uptake did not take place after subjecting to heat-inactivation which occurs with most proteins (5 min, 85°C), suggests that some cytoplasmic protein is involved in the phenomenon observed. Whether this protein would act enzymatically in the polymerization of melanin or by some other type of catalysis is not known. The same must be said about the fixation of polymerized melanin to proteins of the cytoplasmic granules.

Which stage of oxidation of dopa was taken up by the cells, dopachrome or a later step on the way to the subsequent formation of melanin, is obscure. Under the experimental conditions in an atmosphere of nitrogen, no direct aerobic oxidation could have taken place; nevertheless, active pigment-uptake from previously partially autoxidized substrate solutions was obtained. This was seen with autoxidized solutions of dopa, dopamine, and epinephrine. With dopa, dark brown or black melanin-like pigments were observed in the cells, whereas after incubation with epinephrine, yellow or yellow-brown pigmentation was obtained. This was typical also for the iodine-oxidized solutions, where rapid and complete oxidation of the substrates to dopachrome or adrenochrome had been produced. Two explanations for this difference in color are possible. The final melanins produced from dopa and epinephrine may be significantly different in this respect. Alternatively, soluble compounds, like adrenochrome, may be taken up by some cytoplasmic proteins and then not altered, stay yellow, whereas in the case of dopa further oxidation occurs to black dopa melanin.

Present findings indicate that the pigment-production in the intestinal cells, or in skin or in leucocytes, is not highly specific, as has been claimed to be the case for the histochemical dopaoxidase reaction in the skin.

The pigmentation developed in previously autoxidized solutions over a pH range of 5 to 9 or higher. It has also been shown recently in a critical study of the dopa-factor by VAN DUIJN¹⁵ that mushroom-phenoloxidase-oxidized dopa solutions give a similar positive reaction at a low pH (4-6.5).

The epidermis reaction was claimed to be specific for L-dopa and negative for D-dopa (BLOCH and SCHAAF¹⁶); however dopamine, which has no asymmetric carbon atom was reported to give the same reaction (MULZER and SCHMALFUSS¹⁷). In the present study it was found that intestinal cells give the same strong reaction with pre-oxidized epinephrine or its dextro-isomer, suggesting that no such specific phenolase was involved in this step.

The possibility was considered that this reaction could be related to some peroxidase-activity. If in some of the oxidation-steps before or after the formation of dopachrome a peroxide should be formed (MASON⁹), and if these cells should contain peroxidase activity, it is conceivable that an amount of melanin could be formed out of the partly oxidized substrate even in an atmosphere of nitrogen to a sufficient extent to lead to cytoplasmic pig-

mentation. In a few experiments a strong benzidine peroxidase reaction was found with pigmented cells, and also in similar cells of sections not previously treated. This observation needs further confirmation. Peroxidases are known to be HCN-sensitive, whereas the pigment-uptake is very little affected by HCN according to VAN DUIJN¹⁸.

A number of the present observations obviously require further study for their elucidation. It is so far not clear why with incubation media containing tryptamine, which remained clear and visibly colorless for a number of days, similar pigment-uptake was found as with tryptophane, hydroxytryptamine, etc. Reaction media which have been allowed to stand at room temperature for over 2-3 h or overnight have not been checked for bacterial growth, which could possibly account for some of the 'spontaneous' oxidation taking place in them.

Nothing is known regarding the further fate of these pigmented cells, and whether they are capable of eliminating the pigment. It was reported that in cases of melanosis coli of man, ascribed to the prolonged use of cascara sagrada medication (anthraquinone or emodin), the pigmentation was reversible (BOCKUS, WILLARD, and BANK¹⁹).

A re-evaluation of the histochemical dopaoxidase reaction in skin is desirable, according to these results and to those which have been obtained by VAN DUIJN¹⁵. In spite of these differences between the classical melanocytes in the epidermal part of the skin and the cells in the intestine or in the dermis described here, the possibility that some stages of the pigment development and uptake by these cells may be related or identical cannot be ruled out. It would not seem unlikely, that the reaction found in epidermal melanocytes is but a cytologically specialized or adapted form of a more general phenomenon of the possibly non-enzymatic oxidation of dopa, epinephrine, or tryptamines. If this reaction, which is quite rapid, occurs *in vivo*, and if quantitation could be achieved, it might provide information about the oxidative metabolic pathways of the physiologically and pharmacologically important compounds mentioned above.

Zusammenfassung

Gefrierschnitte von frischem oder formalin-fixiertem Dünndarm verschiedener Tierarten werden in teilweise autoxydierten, 2×10^{-3} -molaren Lösungen von Dopamin, Dopa, Adrenalin, Hydroxytryptamin und verwandten Verbindungen bei pH = 8 inkubiert. Gewisse Zellen der tunica propria der Schleimhaut zeigen dann nach relativ kurzer Zeit eine zum Teil sehr starke granuläre Pigmentierung ihres Zytoplasmas.

¹⁸ P. VAN DUIJN, Acta physiol. pharmacol. Neerland 5, 413, 428 (1957).

¹⁹ H. L. BOCKUS, J. H. WILLARD, and J. BANK, J. Amer. med. Ass. 101, 1 (1933).

Corrigendum

G. ZBINDEN und A. STUDER: *Histochemische Untersuchungen über den Einfluss von Iproniazid (Marsilid) auf die durch Reserpin erzeugte Freisetzung von Adrenalin und Noradrenalin aus dem Nebennierenmark*. Exper. 14, fasc. 6, 201 (1958).

Die beiden letzten Sätze der englischen Zusammenfassung müssen richtigerweise wie folgt lauten:

In animals pretreated with equimolar doses of isoniazid, however, histochemical catecholamine reactions show a marked decrease in all cells of the adrenal medulla. These results suggest that monoamine oxidase plays a part in the reserpine-induced release of catecholamine.

¹⁵ P. VAN DUIJN, J. Histochem. Cytochem. 1, 143 (1953); Acta physiol. pharmacol. Neerland 5, 413, 428 (1957).

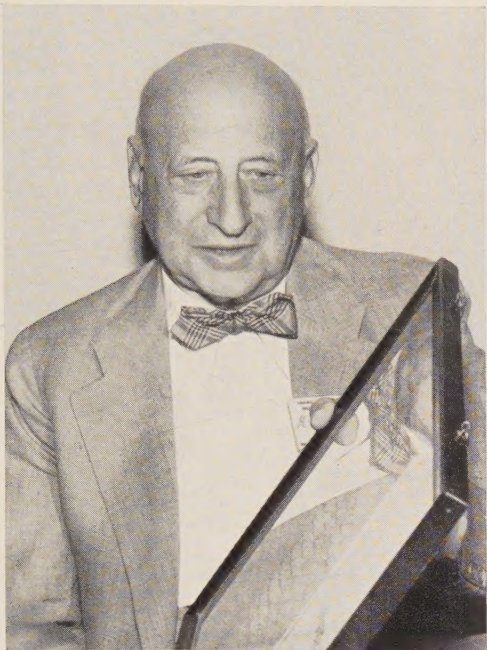
¹⁶ B. BLOCH and F. SCHAAF, Klin. Wschr. 11, 11 (1932).

¹⁷ P. MULZER and H. SCHMALFUSS, Med. Klinik 27, 1099 (1931).

In memoriam Richard Goldschmidt

Redaktionelle Vorbemerkung. Der nachstehende Nekrolog erscheint gleichzeitig in SCIENCE und EXPERIENTIA, damit das universelle Heimatrecht Richard Goldschmidts zum Ausdruck bringend. Die Leser von EXPERIENTIA erinnern sich dankbar der verschiedenen genetischen Aufsätze dieses immer anregenden Geistes, dem unsere Wissenschaft grosse Impulse verdankt.

M.



*Wehe jenen Weisen, die zu stark sind,
der Menge von ihrem Wissen mitzu-
teilen; sie sind ein Fluch, der zu
stark ist, die Reifenden zu verwirren.*

*Confucius
(nicht wörtlich)*

*Den besten Studenten zur Erin-
nerung an*

Richard Goldschmidt

20.6.79.

On April 12, 1958, RICHARD GOLDSCHMIDT was 80 years old. He was recovering from a nearly fatal illness which had struck a few weeks earlier. Letters from friends and well wishers arrived from many lands praising his achievements as a biologist, a zoologist, a geneticist, expressing amazed admiration for his continued scientific productivity, and professing warm personal feelings of affection. That birthday, quietly spent with his family, was a happy day in a period of years in which physical pain and fear that the body might force the mind into inactivity were ever present. Less than two weeks later, on April 24, the end came.

RICHARD GOLDSCHMIDT's ancestors belonged to respected families who had lived for centuries in Frankfurt am Main. He received the excellent education of the Gymnasium and at an early age decided to become a naturalist. In 1896 he entered the University of Hei-

delberg where to use his own words, he 'had such glorious teachers as BÜTSCHLI, GEGENBAUR, KÜHNE, V. MEYER, KOSSEL, ROSENBUSCH'. After a short period in Munich under RICHARD HERTWIG, he returned to Heidelberg and obtained the Ph. D. degree in 1902. From 1903–1913 he worked and taught in Munich. In 1914, GOLDSCHMIDT was selected by BOVERI to join him as a member of 'the newly founded ... wonderful Kaiser Wilhelm Institut für Biologie, Berlin-Dahlem'. Soon after, for reasons of health, BOVERI had to give up his projected move to the Institut. GOLDSCHMIDT accepted his appointment which extended over 22 years, the last 15 of which he served as a director. In 1936, after the passage of the 'Nürnberg Laws', he accepted an invitation to join the Zoology Department of the University of California. 'This turned out to be one of the most happy events of my life, crowned by becoming an American citizen in 1942'. Thus he wrote in an autobiographical sketch filed, by request, with the National Academy of Sciences in Washington (1948). By then he had, according to American custom, included a middle name in his publications: RICHARD BENEDICT GOLDSCHMIDT.

In Berkeley he taught genetics and cytology for more than a decade and uninterruptedly continued his research for 22 years. Reports on experiments and wide ranging books followed one another. Even after his death two papers appeared in print.

GOLDSCHMIDT's work covers nearly 60 years of tireless productivity. When, in 1954, he compiled a list of his 17 books and approximately 250 papers he divided the latter into the following classes: Protozoology (1904 to 1907), Cytology (1902–1950), Embryology (1900 to 1935), Histology and Neurology (1903–1910), Acrania (1905 to 1933), Gynandromorphism (1922–1937), Intersexuality (1911–1951), General Sex Determination, Sex-controlled Heredity (1910–1953), Genetics and Evolution (1911 to 1953), Genetics: Mendelian Analysis and General (1913 to 1954), Physiological Genetics (1916 to 1952), Human Heredity (1927–1953), Biographical, Popular Science, Varia (1916–1953). He listed his books under Technical, Textbooks, Popular and Travel. Among these were such works as *Die quantitativen Grundlagen von Vererbung und Artbildung* (1920), *Mechanismus und Physiologie der Geschlechtsbestimmung* (1920), *Physiologische Theorie der Vererbung* (1927), *Die sexuellen Zwischenstufen* (1931), *Physiological Genetics* (1938), *The Material Basis of Evolution* (1940), *Theoretical Genetics* (1955); *Einführung in die Vererbungswissenschaft* (first edition 1911, fifth edition 1928); *Ascaris, eine Einführung in die Wissenschaft vom Leben* (first edition 1921, third edition 1953); *Neu-Japan* (1927). Translations of his books appeared in English, French, Hebrew, Japanese, Polish, Russian, Spanish, and Yugoslavian. His latest volume, the charming *Portraits from Memory, Recollections of a Zoologist* (1956) is in the process of being translated into German. An autobiography went to press this spring.

GOLDSCHMIDT's influence on the biology of the twentieth century rested on observation and experiment as well as on the theory-building sweep of his imagination. His outstanding experimental accomplishment was the long series of crosses between geographical races of the gypsy-moth *Lymantria*. It led to an analysis of the phenomenon of intersexuality which went far beyond

the framework of classical genetics. He had early trained himself to be a revolutionary of science. He reached his height in his endeavors to build a dynamic physiological genetics on the static and material basis which MENDEL and MORGAN had laid and which he admired as such without reservation. He raised his voice in warning of a too ready acquiescence in apparently established concepts of the gene and some widely held genetic interpretations of evolution. He was willing to face the strong opposition to his unpopular ideas but he lived to see them move into the forefront of contemporary thought.

He lectured before thousands of eager listeners – students, colleagues, men of other professions and interested lay people – in Europe, America, Asia, and Australia. Three periods which he spent in Japan have been historic events in the history of genetics of that country. Among his students, pre- or postdoctoral, were men and women like BUCHNER, RHODA ERDMANN, GARDNER, ALOHA HANNAH-ALAVA, MASUI, MINOUCHI, NACHTSHEIM, POPOFF, SEILER, and SÜFFERT. In a wider sense, the circle of this disciples encompasses biologists everywhere.

GOLDSCHMIDT's interests went greatly beyond his 'beloved zoology'. Until middle age he excelled in various sports. He molded his life after Goethe and only recently, he and his wife systematically read again all of Goethe's works. His main avocation was that of a connoisseur and collector of art. The home which he and Mrs. GOLDSCHMIDT created became a treasure house of paintings, statues, and other objects, each of which had been acquired with love and insight. The art of the orient was his particular area of pleasure and understanding. The many visitors which Mrs. GOLDSCHMIDT and he welcomed gained lasting impressions from these occasions.

The life of RICHARD GOLDSCHMIDT was woven into the framework of this time. As a young man he served in the army of his native country. When World War I kept him in the United States where he had come from Japan, he first enjoyed the hospitality of American scientific institutions; but when America entered the war, his professed loyalty to Germany led to his internment as an enemy alien. When in 1919 he returned to the Kaiser-Wilhelm-Institut he soon rose to a commanding position among the great scientists who upheld the tradition of intellectual achievements of their country. When that tradition broke in 1933 he had to prepare to leave Germany and build up anew his life's activities and to take roots again in a different country. Bitter, he resumed his tasks at the age of 58 years. His reception in America, though warm in a personal way, was cold scientifically. The breadth of his biological wisdom was appreciated little and the iconoclasm of his views regarded as the irritating fancy of a man of the past. But slowly recognition came again. Following the numerous honors which had earlier been RICHARD

GOLDSCHMIDT's share, election to the National Academy occurred in 1947 when he was 71 years old. In 1950 the Genetics Society of America celebrated the Golden Jubilee of Genetics and GOLDSCHMIDT was asked to give the opening address. The Cold Spring Harbor Symposium on Genes and Chromosomes in 1951 saw him as the first speaker. In 1953 he was elected President of the 9th International Congress of Genetics in Bellagio.

A reconciliation came in his attitude to Germany. He had never broken the bonds of personal friendship and he and his wife had sent many *Care* and other packages to the devastated post war country. While he could not bring himself to visit his homeland again, he was willing to accept on the occasion of his 75th birthday, honorary membership in the Deutsche Zoologische Gesellschaft and an honorary degree from the Freie Universität Berlin. And in his last year he addressed a German audience when he spoke with his Frankfurt accent over the Süddeutsche Rundfunk on 'Aus der Geschichte der Vererbungswissenschaft'.

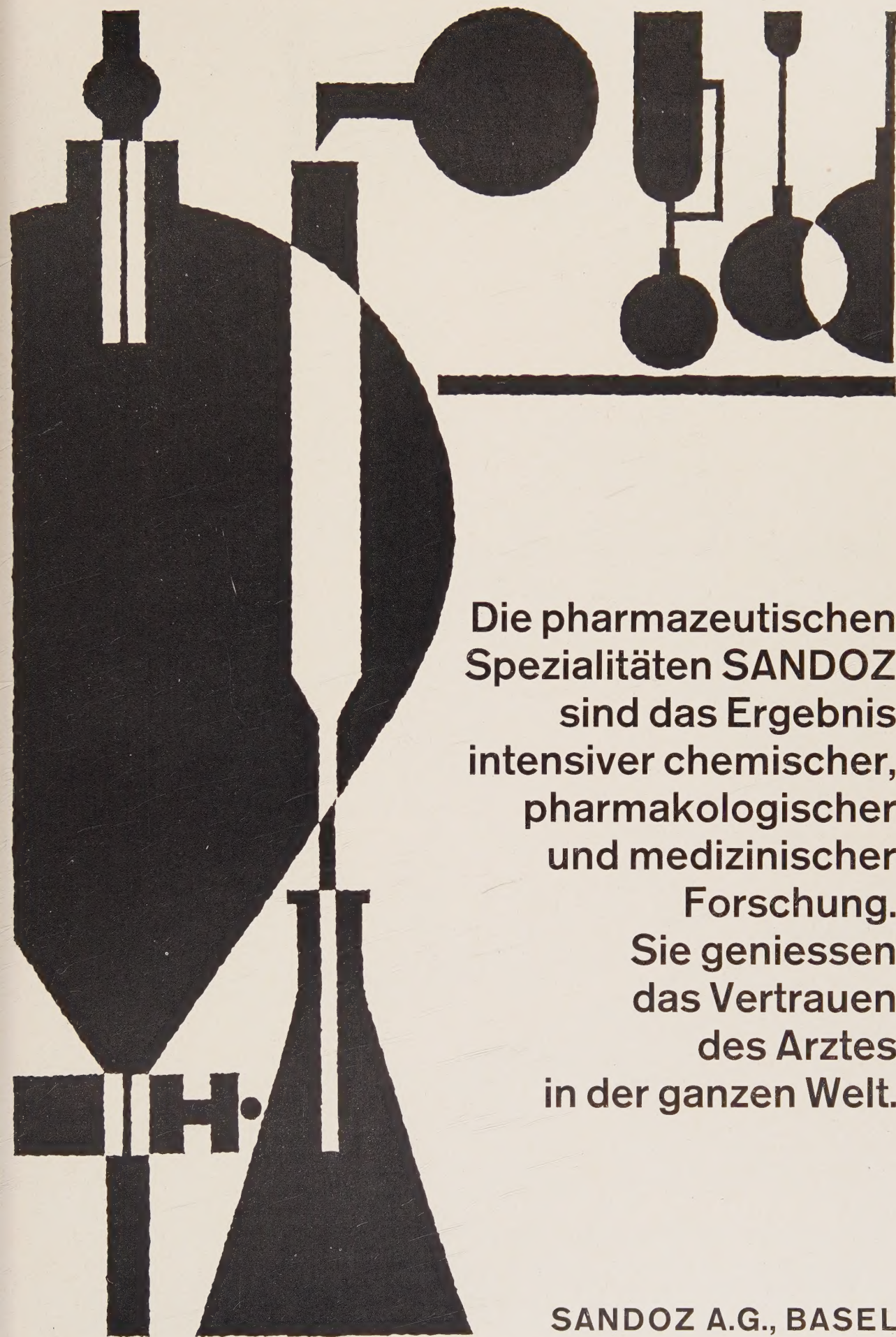
He never attained old age. He could have been the grand old man of more than a half century of biology – and yet he never was given that *retrospective* title. People corresponded with him and visited him as the *contemporary* leader.

He knew his worth – how could he help it. He openly and with relish levelled his critique against those whose view he opposed. Only a few years ago, he chose the pungent title 'Pricking a bubble' for a polemic. But he could also write about his non-conformist views under the modest title 'Evolution, as viewed by one geneticist'. He charmed his adversaries when they met in person, with his ability to divorce the intellectual points of divergence from the appreciation of the fellow scientist. He inspired awe by his achievements and by an often forbidding appearance. Yet his kindness and solicitude became apparent to those who approached him. His assistants, he treated as fellow scientists. To his friend the late Oberpräparator AIGNER he dedicated a paper in Naturwissenschaften. 'In Ph. D. examinations' one colleague wrote him, 'we depended on you to bring forth the most favorable side of the candidates' performance, and I always thought of you as the aspiring students' 'Advokat'. Another letter read: 'How impressed I was (on the boat to Stockholm in 1948) with your thoughtful interests in us young unknowns!!' 'The guardian angel of the small people' he was called in 1927.

RICHARD GOLDSCHMIDT formed his existence into a piece of art. It had the classical features of comprehensiveness, depth, and achievement. It had the modern features of expressionism where emphasis broke the bounds of beauty conceived too narrowly. Like any mortal he did not attain perfection, nor did he aspire to it. Like few mortals, he achieved greatness.

Berkeley, California.

CURT STERN



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